University of Alberta

Characterization of Mitochondrial Fatty Acylation and Identification of a New Fatty Acylated Protein: Carbamoyl Phosphate Synthetase 1

by

Carrie-lynn Mary Soltys ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

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Carrie-lynn Soltys

175 Mark Street
Sault Ste. Marie, Ontario
P6A 3M4

Date: Aug. 11/99

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of Mitochondrial Fatty Acylation and Identification of a New Fatty Acylated Protein: Carbamoyl Phosphate Synthetase 1** submitted by **Carrie-lynn Mary Soltys** in partial fulfillment of the requirements for the degree of Master of Science.

Dr. Luc G. Berthiaume

Dr. Dennis E. Vance

Dr. Vickie Baracos

Date: Aug 10/99.

To my family,

Just for today - I will live through the next 12 hours and not try to tackle all of life's problems at once.

Just for today - I will improve my mind. I will learn something useful. I will read something that requires thought and concentration.

Just for today - I will be agreeable. I will look my best, moderate my voice, be courteous and considerate.

Just for today - I will not find fault with friend, relative or colleague. I will not try to change or improve anyone but myself.

Just for today - I will do a good turn and keep it a secret. If anyone finds out, it won't count.

Just for today - I will have a program. I might not follow it exactly, but I will have it. I will save myself from two enemies --- hurry and indecision.

Just for today - I will do two things I don't want to do, just because I need the discipline.

Just for today - I will believe in myself. I will give my best to the world and feel confident that the world will give its best to me.

ABSTRACT

Protein fatty acylation is involved in dynamic regulation of mitochondrial metabolism. Studies have demonstrated acylated proteins in COS-7 and rat liver mitochondria. Acylation of the mitochondrial enzyme methylmalonyl semialdehyde dehydrogenase occurs on its active site cysteine, inhibiting its activity. This thesis identifies a new fatty acylated mitochondrial protein: carbamoyl phosphate synthetase 1 (CPS 1) and describes the potential role of acylation in mitochondrial metabolic regulation. [^{125}I]iodopalmitate labeling of rat tissue, mouse tissue and hepatocyte mitochondria indicated differing mitochondrion acylation profiles, both between tissues and cell types. Acylation occurred within the mitochondrial matrix and inner membrane. An acylated 165 kDa rat liver mitochondrial protein was purified and identified as carbamoyl phosphate synthetase 1 (CPS 1). CPS 1 palmitoylation occurred via a covalent hydroxylamine sensitive thioester bond. Physiological concentrations of palmitoyl-CoA inhibited CPS 1 activity. Regulation of CPS 1 enzymatic activity by fatty acylation suggests long chain fatty acyl-CoAs may mediate metabolic cross-talk between catabolic degradation of amino acids and fatty acids.

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LIST OF ABBREVIATONS

AAT	ADP/ATP tranlocase
ACC	acetyl-CoA carboxylase
ADP	adenosine 5' diphosphate
AGA	*N*-acetyl *L*-glutamate
APT1	acyl protein thioesterase 1
ATP	adenosine 5' triphosphate
BCKA	branched-chain α-ketoacids
BSA	bovine serum albumin
Ci	Curie
CL	citrate lyase
cm	centimeter (10^{-2} meters)
CoASH	Coenzyme A
COS-7	CV-1 origin, SV40 transformed monkey kidney cell line
CPS 1	carbamoyl phosphate synthetase 1
CPT I	carnitine palmitoyl transferase I
CPT II	carntine palmitoyl transferase II
CMC	critical micelle concentration
°C	degree(s) Celsius
ddH_20	double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide

DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N,N' tetraacetic acid
ETC	electron transport chain
FAD	flavin adenine dinucleotide
$FADH_2$	reduced FAD
FAS	fatty acid synthetase
FPLC	fast performance liquid chromatography
FSBA	flurosulfonylbenzoyladenosine (ATP analog)
g	gram
g	acceleration due to gravity
GAT	glutamine amido transferase domain
GluDH	glutamate dehydrogenase
GPI	glycosylphosphatidylinositol structure
HEPES	N-2-Hydroxyethylpiperazine-N'-Z-ethanesulfonic acid
$HC0_3^-$	bicarbonate
HPLC	high performance liquid chromatography
IC16	iodopalmitate analog
IM	isolation medium
IMM	inner mitochondrial membrane
IMS	intermembrane mitochondrial space

kDa	kiloDalton
K_m	Michaelis-Menton constant
K-MES	2-[N-Morpholino]ethanesulfonic acid neutralized with KOH
K-MOPS	3-[N-Morpholino]propanesulfonic acid
l	liter
LCAD	long-chain acyl CoA dehydrogenase
M	molar (moles per liter)
MAB	mitochondrial acylation buffer
MCAD	medium-chain acyl CoA dehydrogenase
McA RH7777	McArdle rat hepatoma 7777
mg	milligram (10^{-3} grams)
min	minute
ml	milliliter (10^{-3} liters)
mM	millimolar (10^{-3} moles per liter)
MMSDH	methyl malonyl semialdehyde dehydrogenase
MMS	methyl malonate semialdehyde
MS	malonate semialdehyde
n=	number of samples
NAD	nicotine adenine dinucleotide
NADH	reduced NAD
ND	not determined
NEM	N-ethyl maleimide

NH_3	ammonia
NMT	N-myristoyltransferase
NP-40	Nonidet P40
OA	oxaloacetate
OMM	outer mitochondrial membrane
PAGE	polyacrylamide gel electrophoresis
PAT	palmitoyl acyl transferase
PDH	pyruvate dehydrogenase
pH	-log of the concentration of H^+ in solution
Pi	free phosphate
PMSF	phenylmethylsulfonyl fluoride
PM	plasma membrane
PTE	palmitoyl thioesterase
PTK	protein tyrosine kinase
rpm	revolutions per minute
SCAD	short-chain acyl CoA dehydrogenase
S.D.	standard deviation
SDS	sodium dodecyl sulfate
STE	sterile Tris-EDTA
TCA	tricarboxylic acid cycle
TEMED	tetramethylethylenediamine
TG	triglyceride
TLC	thin layer chromatography

Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
U	Unit
UDGPT	UDP-glucuronosyl transferase isoforms
μCi	microcurie (10^{-6} curies)
μg	microgram (10^{-6} grams)
μl	microliter (10^{-6} liters)
μM	micromolar (10^{-6} molar)
VLCAD	very-long-chain acyl CoA dehydrogenase
v/v	volume per unit volume
w/v	weight per unit volume

1.0 INTRODUCTION

1.1 Overview

At the protein level, strategies to secure regulation include covalent modifications and subsequent changes in conformation or subunit number. Although protein phosphorylation represents the majority of regulatory covalent modifications observed, the covalent modification of proteins by lipids (protein lipidation) is increasing in importance. The lipidation of proteins greatly alter their physical and functional properties and covalently attached lipids are important components of a diverse array of cellular proteins. There are now more than 200 known lipidated proteins and the number is rapidly growing (Casey, 1995; Milligan *et al.*, 1995; Resh, 1994; Dunphy and Linder, 1998).

1.2 Protein Lipidation

Each different lipid modification can impart distinct functional attributes to their protein hosts depending on the type of lipidation furnished by the protein. Diversity is provided by four major categories of protein lipidation (Table 1.1). These are: prenylation, glypiation, cholesteroylation and fatty acylation (Casey, 1995; Porter *et al.*, 1996; Bhatnagar and Gordon, 1997). Prenylation is the covalent modification of proteins by the isoprenoids, farnesyl (C15:0) and geranylgeranyl (C20:0). These lipids are attached posttranslationally to one or more cysteine residues at or near the C-terminus of the protein via a stable thioether bond. Glypiation is the covalent modification of proteins at their C-terminus by glycosylphosphatidylinositol structures (GPI) (complex structure containing ethanolamine, sugars and phosphatidylinositol). Cholesteroylation

is the covalent modification of proteins by cholesterol is known to occur on only one type of protein family so far, the Hedgehog (Hh) secreted signal proteins (Porter *et al.*, 1996). Finally, protein fatty acylation is the covalent modification of proteins by fatty acids and it is divided into two categories: myristoylation and palmitoylation (Bhatnagar and Gordon, 1997). Most important to my thesis, is the covalent modification, fatty acylation.

1.3 Fatty Acylation

1.3.1 Myristoylation

Myristoylation is characterized by the co-translational attachment of the saturated 14 carbon myristate (C14:0) to the N-terminal glycine residue of a protein via a stable amide bond (Table 1.2). Due to the high stability of the amide bond, myristoylation appears to be an irreversible modification although there have been exceptions (da Silva and Klein, 1990). The consensus sequence of myristoylation has been well defined;

$$MG_2XXX(S/T/C)_6XX$$

where the initiator methionine is removed *in vivo*, M-methionine, G=glycine, X=any small aliphatic amino acid, S=serine, T=threonine and C=cysteine.

A prerequisite for myristoylation is the removal of the initiator methionine of the protein by a cellular methionyl aminopeptidase, thus exposing the N-terminal glycine residue. Then, *N*-myristoyltransferase (NMT) catalyzes the transfer of myristate to the N-terminal glycine. NMT has been extensively characterized (Johnson *et al.*, 1994), and while myristoyl-CoA is the preferred fatty acyl-CoA substrate, it has been demonstrated that smaller substrates (C3-

C13) can be transferred onto peptides by NMT (Heuckeroth *et al.*, 1988b; Kishore *et al.*, 1991). Conversely, larger CoA derivatives (C16:0 or greater) can bind to NMT but fail to be transferred to the protein (Gordon *et al.*, 1991; Johnson *et al.*, 1994).

The glycine residue at position 2 of the protein, immediately adjacent to the initiator methionine, is essential for catalysis since substitution by any other amino acid prevents myristoylation (Resh, 1990; Gottlinger *et al.*, 1989; Bryant and Ratner, 1990). In addition either serine, threonine or cysteine are preferred at position 6 (Gordon *et al.*, 1991; Johnson *et al.*, 1994). Positively charged residues at position 7 to 9 also increase the affinity of the myristate moiety while large bulky hydrophobic residues (e.g. Tyr or Phe) or proline at position 3 or 5 decrease affinity (Gordon *et al.*, 1991). While myristoylation has been shown to influence protein conformation (Nadler *et al.*, 1993; Zheng *et al.*, 1993), as well as protein-protein interactions (Takasaki *et al.*, 1999; Chow *et al.*, 1987), and protein-ligand interactions (Franco *et al.*, 1995), the most obvious function of the myristoyl moiety is to mediate membrane binding. Evidence of the later function has been presented for several proteins through the subcellular localization of myristoylated deficient mutants (Busconi and Michel, 1994; Yu and Felsted, 1992; David-Pfeuty *et al.*, 1993; Hallak *et al.*, 1994a). It has been demonstrated that the Gibbs free binding energy of myristoylated glycine to membranes (approximately 8 kcal/mol) provides just enough hydrophobicity to cause transient association of the myristoylated protein with the membrane (Peitzsch and McLaughlin, 1993; Silvius and L'Heureux, 1994), therefore, myristoylation

alone is not sufficient to confer stable membrane binding. Typically a second type of interaction contributes to stable membrane binding such as additional lipid modifications, the presence of a hydrophobic region on the protein or a string of positively charged residues on the protein that may interact with negatively charged phospholipids.

1.3.2 Palmitoylation

Palmitoylation is characterized by the post-translational attachment of the saturated 16 carbon fatty acid palmitate (C16:0) to one or more cysteine residues of a protein via a thioester bond (Table 1.2). Palmitoylation can also occur on threonine or serine residues but this is not as common. For protein palmitoylation to occur, palmitate must be activated in the form of its coenzyme A derivative, palmitoyl-CoA (Berger and Schmidt, 1984; Bizzozero and Lees, 1986). Palmitoylation is a reversible modification (Mumby *et al.*, 1994) and due to its reversible nature, it has been recently shown to regulate the subcellular localization of several proteins involved in signal transduction processes (Casey, 1995; Milligan *et al.*, 1995; Dunphy and Linder, 1998). The labile thioester linkage permits repeated cycles of palmitoylation and depalmitoylation of a protein and, as such, acts as a molecular switch for a protein during its existence in a cell (James and Olson, 1990) (Fig. 1.1).

In contrast to N-myristoylation and C-terminal prenylation and glypiation, palmitoylation has been shown to occur in variable regions of the protein. Palmitoylation can be found at N-terminal myristoylation sites ($MG_2CXXX(C/S/T)_6XX$), in internal regions of proteins, within the body of

transmembrane proteins in proximity to their membrane spanning domains, near C-terminal prenylation sites, and near or in domains of several proteins including viral membrane envelope glycoproteins (Gonzola and Linder, 1998; Berthiaume *et al.*, 1994; Bouvier, 1995; Caballero *et al.*, 1998). However, palmitoylation of the cysteine within the N-terminal myristoylation sequence MG_2C (the initiator methionine becomes removed *in vivo* by a cellular methionyl aminopeptidase), is shared by 7 of 9 members of the Src family of protein tryrosine kinases (PTKs) and several of the α subunits of the heterotrimeric G proteins (Milligan *et al.*, 1995; Resh, 1994). In many cases, myristoylation is a prerequisite for palmitoylation to occur (Alland *et al.*, 1994; Mumby *et al.*, 1994). Interestingly, it has also been observed that acylated cysteine residues are frequently preceded and/or followed by basic amino acids (Bizzozero *et al.*, 1994). Therefore, the positive charges of the amino acids in the proximity of the palmitoylated cysteine may act as a recognition site for the PAT or the positively charges amino acids could act as a binding site for the negatively charged fatty acyl-CoA.

Myristoylation or prenylation alone can barely provide enough hydrophobicity to anchor proteins onto membranes (Peitzsch and McLaughlin, 1993; Silvius and L'Heureux, 1994). Palmitate confers 15X more membrane binding affinity than myristate (Smith and Powell, 1986). As such, palmitoylation nicely complements myristoylation and prenylation of proteins to stably anchor these proteins into membranes. Interestingly in many cases, myristoylation or prenylation are prerequisites for palmitoylation to occur and

mutations abolishing myristoylation or prenylation has also been shown to abolish palmitoylation (Wolven *et al.*, 1997).

The molecular machinery involved in palmitoylation has yet to be fully identified, but there is evidence for enzymes that catalyze either the addition or removal of long chain fatty acids from cysteine residues (Schlesinger *et al.*, 1993; Berthiaume and Resh, 1995). The enzyme responsible for palmitoylation is referred to as protein fatty acyltransferase (PAT) or protein fatty S-acyltransferase. However, another term frequently used for palmitoylation is S-acylation due to PAT's loose specificity, since it has been demonstrated that other long (>C16:0) fatty acyl-CoA chains may substitute for palmitoyl-CoA (Bizzozero *et al.*, 1994; Schlesinger *et al.*, 1993; Hallak *et al.*, 1994b). Hallak *et al.*, (1994b) also demonstrated that unsaturated fatty acids can also be utilized as substrates for PAT and therefore transferred to proteins.

Since no overall consensus sequence has been found at the palmitoylation site, more than one PAT may exist. Membrane associated protein S-acyltransferase activities have been partially purified (Berthiaume and Resh, 1995; Dunphy *et al.*, 1996; Veit *et al.*, 1996, 1998), but their relevance towards the regulation of fatty acylation remains to be elucidated. A protein acyltransferase that palmitoylates farnesylated Ras proteins was purified (Lui *et al.*, 1996) but its ability to palmitoylate Ras was later determined to be due to the activity of a thiolase A protein, and its relevance in the palmitoylation of Ras has yet to be determined.

The enzyme palmitoyl thioesterase (PTE) is responsible for cleaving palmitate from modified proteins. A PTE that deacylates Ras proteins and the α subunits of G proteins *in vitro* has been purified (Camp and Hofmann, 1993; Camp *et al.*, 1994) but was shown to be a secreted protein. Therefore, Ras proteins and Gα subunits, which are located on the cytoplasmic layer of the plasma membrane, are unlikely physiologic substrates for this enzyme. A cytosolic acyl-protein thioesterase (APT1) has also recently been cloned (Duncan and Gilman, 1998), but its relevance to fatty acylation regulation remains to be elucidated.

It is important to note that fatty acylation of cysteine thiols has been shown to occur non-enzymatically. Several proteins and peptides can be palmitoylated in the absence of PAT using palmitoyl-CoA as the donor. Proteins shown to be autoacylated are: G protein α subunits (Duncan and Gilman, 1996), mylein proteolipid protein (PLP) (Bizzozero *et al.*, 1987; Bizzozero *et al.*, 1990), mylein P_o glycoprotein (Bharadwaj and Bizzozero, 1995), rhodopsin (O'Brien *et al.*, 1987), Semliki Forest virus E2 glycoprotein (Berger and Schmidt, 1984), UDP-glucuronosyl transferase isoforms (UDGPT) (Yasmashita *et al.*, 1995), porcine pancreatic phospholipase A_2 (Tomasselli *et al.*, 1989) and methylmalonyl semialdehyde dehydrogenase (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). While the physiological relevance of this process is not yet clear, autoacylation of the myelin proteolipid protein, myelin P_o glycoprotein and the Giα1 protein occur at the same sites as those that become palmitoylated enzymatically *in vivo* (Bizzozero *et al.*, 1987; Bharadwaj

and Bizzozero, 1995; Duncan and Gilman, 1996). This observation raises the question for the requirement of an enzyme. Autoacylation, like enzymatic palmitoylation was also shown to be promiscuous with regard to the identity of the fatty acid substrate but more studies need to be performed *in vivo* (Hallak *et al.*, 1994b; Duncan and Gilman, 1996).

Functions of protein palmitoylation include subcellular targeting (or localization) proteins to membranes and organelles (Wolven *et al.*, 1997; Gonzola and Linder, 1998; Bijlmakers *et al.*, 1997; Melkonian *et al.*, 1999), sorting of proteins (Breuer and Brulke, 1998), mediation of protein-protein interactions involving activation (Ponimaskin *et al.*, 1998) or inhibition (Nakamura *et al.*, 1998; Sudo *et al.*, 1992), regulation of membrane receptor endocytosis and downregulation (Bouvier, 1995), regulation of enzymatic activity (Berthiaume *et al.*, 1994), and virus budding (Ivanova and Schlesinger, 1993). Palmitoylated proteins have been found in the plasma membrane, mitochondria, Golgi/*trans* -Golgi Network (TGN), endosomes, and even the extracellular milieu (Berthiaume *et al.*, 1994; Schroeder *et al.*, 1996; Breuer and Brulke, 1998; Pepinsky *et al.*, 1998).

As previously stated, for protein palmitoylation to occur, the fatty acid palmitate needs to be activated in the form of its coenzyme A derivative, palmitoyl-CoA. Interestingly, palmitoyl-CoA the acyl donor for both enzymatic and nonenzymatic (autoacylation) palmitoylation of proteins, inhibits several mitochondrial enzymes including: rat adipocyte pyruvate dehydrogenase (PDH) (Moore *et al.*, 1992), rat liver ADP/ATP translocase (AAT) (Morel *et al.*, 1974),

bovine liver glutamate dehydrogenase (GluDH) (Kawaguchi and Bloch, 1976), and bovine liver methylmalonyl semialdehyde dehydrogenase (MMSDH) (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). The possibility that protein fatty acylation may serve as a regulator of mitochondrial function, is of particular interest and is the focus of my thesis.

1.4 Mitochondria

1.4.1 Structure and Function of the Mitochondria

The mitochondrion is a double membrane structure that consists of the outer membrane (OMM), the inner membrane (IMM), the intermembrane space (IMS), and the matrix (MM) (Fig. 1.2). The outer mitochondrial membrane contains the transmembrane protein porin which allows free diffusion of ions and water-soluble metabolites with molecular weights less than ~10 kDa across the membrane. The inner mitochondrial membrane is highly folded to form special structures called cristae which greatly increase its surface area to 3-5 times greater than that of the outer membrane. While the inner membrane is impermeable to ions and most metabolites, it is permeable to uncharged molecules and substances crossing the inner membrane must be transported by specific membrane-spanning transporters. The area between the inner and outer mitochondrial membrane is referred to as the intermembrane space and its composition in small molecules is the same as the cytosol due to the permeability of the outer membrane to small molecules. The matrix, an aqueous almost gel-like phase due to its high protein concentration, is completely enclosed by the inner membrane.

The inner membrane and the matrix contain components of the protein import apparatus. The inner membrane also contains the components that carry out oxidative phosphorylation; the electron transport chain (respiratory chain) and the ATP synthase (FoF1-ATPase, but some subunits extend into the matrix). Contents of the matrix include the pyruvate dehydrogenase complex, the enzymes of the citric acid cycle (tricarboxylic acid cycle (TCA)) (the succinate dehydrogenase complex of the citric acid cycle is located in the inner membrane) generating reduced intermediates such as NADH that are reoxidized by the electron transport chain (ETC), most of the enzymes that catalyze fatty acid β-oxidation and two of the five enzymes involved in the urea cycle with the rest residing in the cytosol.

1.4.2 β-Oxidation

Mitochondria play a central role in the regulation of cell death, or apoptosis (Green and Reed, 1998), are important in evolutionary biology, and essential for cell viability. In addition, mitochondria are in fact the main organelle responsible for breaking down fatty acids and amino acids to generate energy in the form of ATP. β-oxidation of fatty acids is an important pathway of energy metabolism in mammals, especially during starvation. During the early stages of fasting, the body draws selectively on its supply of energy in the form of triacylglycerol in adipose tissue, sparing at first the breakdown of vitally needed proteins (Moran *et al.*, 1994). In order to generate energy, hydrolyzed fatty acids originating from triacylglycerol stores must enter the β-oxidation cascade in the mitochondria. Therefore, fatty acids in the cytosol

are converted to thioesters of CoA by four different acyl-CoA synthetases that have specificity's for fatty acids with chain lengths that are short (<C6), medium (C6-C12), long (>C12) or very long (>C16). But the fatty acyl-CoAs that are formed in the cytosol cannot freely cross the inner mitochondrial membrane to the mitochondrial matrix, where the reactions of β-oxidation occurs. Therefore, a transport system carried out by carnitine palmitoyl transferase I (CPT I), localized on the outer mitochondrial membrane, carnitine:acylcarnitine translocase and carnitine palmitoyl transferase II (CPT II) (both localized to the inner mitochondrial membrane), is required to shuttle the fatty acids across the inner mitochondrial membrane. Fatty acyl-CoAs in the mitochondrial matrix can enter β-oxidation and be converted to acetyl-CoA. More NADH is generated by acetyl-CoA produced from β-oxidation entering the citric acid cycle. NADH produced from the citric acid cycle and β-oxidation enter the ETC to generate ATP by oxidative phosphorylation.

Branched chained α-ketoacids (BCKA) derived from the essential amino acids (valine, leucine and isoleucine), are metabolized in the mitochondria (Johnson and Connelly, 1972). These substrates are important for energy generation and carbohydrate and ketone body synthesis (Goldberg and Chang, 1978). It has been demonstrated that octanoate and palmitate inhibit BCKA oxidation (Bremer and Davis, 1978; Williamson *et al.*, 1979; Buffington *et al.*, 1979) suggesting a metabolic cross-talk between the BCKA and fatty acid degradation pathways. NADH, $FADH_2$ and acetyl-CoA are produced upon oxidation of BCKAs (from leucine) or propionyl-CoA (from valine and

isoleucine) (Williamson *et al.*, 1979). Interestingly, β-oxidation of fatty acids is inhibited by high NADH/NAD^+, FADH/FAD^+ and acetyl-CoA/CoASH ratios (Schulz, 1985; Latipaa *et al.*, 1986; Hochachka *et al.*, 1977; Whitmer *et al.*, 1978; Olowe and Schulz, 1980). In addition, propionyl-CoA has been shown to inhibit β-oxidation (Brass *et al.*, 1986; Brass, 1986; Glasgow and Chase, 1976; Brass and Beyerinck, 1988).

The first step of mitochondrial fatty acid β-oxidation is mediated by a group of chain-length specific acyl-CoA dehydrogenases including: very-long-chain (VLCAD), long-chain (LCAD), medium-chain (MCAD), and short-chain (SCAD) acyl-CoA dehydrogenases (Moran *et al.*, 1994). Likewise, to derive energy, several amino acids are also catabolized in the mitochondria by a series of dehydrogenases (Salway, 1994). Interestingly, genetic disorders causing deficiency in fatty acyl-CoA dehydrogenases have been shown to result in the disease features of hypoglycemia, hyperammonemia, tissue fatty change, hypoketonemia, carnitine deficiency and organic acidemia (Roe *et al.*, 1995). Acute-clinical episodes which mainly occur in young children are accelerated by fasting and are often fatal (Roe *et al.*, 1995). These toxic effects may be attributed in part to the accumulation of long chain fatty acyl-CoAs within the mitochondria.

1.4.3 Urea Cycle

The urea cycle in the liver plays an essential role in human as well as other ureotelic organisms to efficiently remove toxic ammonia from body tissues. Excess nitrogen resulting from the metabolic breakdown of amino acids passes

from the liver through the bloodstream to the kidney where it is excreted in the urine in the form of urea.

Mitochondrial glutamate dehydrogenase (GluDH) catalyzes the oxidative deamination of glutamate to ammonia which becomes incorporated into urea in five steps. The nitrogen atom of the first product formed in the urea cycle, carbamoyl phosphate, becomes incorporated into urea in the four reactions of the urea cycle. Two of these reactions occur in the hepatocyte mitochondria while the other three occur in the cytosol. Ammonia and aspartate are the precusors of the two nitrogen atoms of urea. The carbon atom of urea is derived from bicarbonate. The overall reaction for urea synthesis is

$NH_3 + HCO_3^- + \text{Aspartate} + 3ATP \rightarrow \text{Urea} + \text{Fumarate} + 2ADP + 2P_i + AMP + PP_i$

Congenital deficiency in any of the urea cycle enzymes results in the accumulation of ammonia. Ammonia toxicity is manifested by altering energy metabolism due to inhibition of fatty acid oxidation, leading in turn to a decrease in both free coenzyme A (CoASH) and adenosine triphosphate (ATP) (Maddaiah, 1985; Maddaiah and Miller, 1989; Ratnakumari *et al.*, 1992).

1.5 Fatty Acylation in Mitochondria

1.5.1 Methylmalonyl Semialdehyde Dehydrogenase

In addition to playing a major role in signal transduction, protein fatty acylation may also play a role in mitochondrial metabolic regulation (Berthiaume *et al.*, 1994). Recently, the mitochondrial protein methylmalonyl semialdehyde dehydrogenase (MMSDH), an enzyme of the distal part of the valine and pyrimidine catabolic pathways, was shown to be fatty acylated on its

active site cysteine residue (Cys319) thereby inhibiting its enzymatic activity (Berthiaume *et al.*, 1994; Deichaite *et al.*, 1993). MMSDH catalyzes the oxidative decarboxylation of malonate and methylmalonate semialdehydes (MS and MMS) to acetyl-CoA and propionyl-CoA, respectively. It was demonstrated that MMSDH's fatty acylation varied with energy level, was reversible and specific for long chain fatty acyl-CoAs (Berthiaume *et al.*, 1994). The fatty acylation of MMSDH required only the fatty acyl-CoA and seemed to be autocatalytic *in vitro* in apparently pure preparations of MMSDH. In support of the autocatalytic mechanism, recombinant MMSDH prepared from *E. coli* lysates were readily fatty acylated in the presence of [^{125}I]iodopalmitoyl-CoA (L.G.B. unpublished).

Based on the above observations and current literature (Williamson *et al.*, 1979; Schulz, 1985; Latipaa *et al.*, 1986; Hochachka *et al.*, 1977; Whitmer *et al.*, 1978; Olowe and Schulz, 1980; Brass *et al.*, 1986; Brass, 1986; Glasgow and Chase, 1976; Brass and Beyerinck, 1988), Berthiaume and coworkers (1994) proposed a model in which the fatty acylation of MMSDH could act as a novel mode of regulation of its enyzymatic activity within the mitochondria, suggesting the possibility of a metabolic cross-talk between amino acid and fatty acid catabolic pathways (Fig. 1.3). In mitochondria, enzymes that are part of different catabolic pathways must compete with each other for a common pool of CoASH, NAD^+ and FAD^+ cofactors and for a common electron transport chain (Brass and Beyerinck, 1988). As such, the variable availability of such cofactors, appropriate catabolites, and competing enzymes can affect the rate of

a given catabolic pathway. However, little is known about the coordination mechanisms which are responsible for the regulation of degradation of these catabolic substrates. MMSDH belongs to a group of NAD^+ dependent dehydrogenases which have been shown to increase the NADH/NAD^+ ratio and therefore capable of inhibiting or decreasing fatty acid β-oxidation by competing for key cofactors. When long chain fatty acyl-CoA thioesters accumulate within the mitochondria, MMSDH or other dehydrogenases could be subjected to negative regulation by fatty acylation, allowing β-oxidation of fatty acids to proceed (Berthiaume *et al.*, 1994). MMSDH contributes to increasing the NADH/NAD^+ and acetyl-CoA/CoASH ratios and both products of the MMSDH enzymatic reaction are also inhibitors of the β-oxidation pathway (Schulz, 1985; Latipaa *et al.*, 1986; Hochachka *et al.*, 1977; Whitmer *et al.*, 1978; Olowe and Schulz, 1980; Brass *et al.*, 1986; Brass, 1986; Glasgow and Chase, 1976; Brass and Beyerinck, 1988) making it a good candidate for negative regulation by fatty acylation. Fatty acids could act like an "ON/OFF" switch. Thus, MMSDH would be turned "ON" (not fatty acylated) only when the cell requires its activity to produce metabolic energy and turned "OFF" (fatty acylated) when it is not required. In this way, the cell could continuously sense levels of fatty acids and amino acids in order to commit one or the other for the generation of energy. The molecular fatty acid modification, or "molecular switch" would therefore be a means of communication between the different metabolic pathways involved.

1.5.2 Glutamate Dehydrogenase

Bovine liver GluDH is a 332 kDA mitochondrial matrix protein that catalyzes the reversible oxidative deamination of L-glutamate to 2-ketoglutarate and ammonia (Golden and Frieden, 1971).

L-glutamate + $NAD(P)^+$ + H_20 <------> 2-Ketoglutarate + NH_3 + NAD(P)H

Glutamate dehydrogenase is a branch point between carbon and nitrogen metabolism. It is an allosteric enzyme and is inhibited by both GTP and high concentrations of NADH, activated by ADP and AMP (Golden and Frieden, 1971). In its active form the enzyme is composed of six identical subunits (Smith *et al.*, 1975). In addition, Kawaguchi and Bloch (1976) demonstrated that bovine liver GluDH enzymatic activity was inhibited by micromolar concentrations of palmitoyl-CoA. Palmitoyl-CoA was shown to convert the GluDH to enzymatically inactive dimeric subunits and bind tightly to the dissociated enzyme. Berthiaume and coworkers (1994) demonstrated that bovine liver GluDH bound fatty acid covalently and that fatty acylation was prevented by preincubation of the enzyme with N-ethyl maleimide (NEM), a cysteine alkylating agent, thereby suggesting a cysteine residue is involved in its acylation but the fatty acylated cysteine has not been identified. In contrast to the mitochondrial enzyme MMSDH, bovine liver GluDH does not utilize an active site cysteine residue to a form a covalent intermediate during catalysis, and its catalytic mechanism is rather complex (Hudson and Daniel, 1993). In addition, the regulation of GluDH is also complex, being regulated differently by various cofactors and nucleotides (activators and inhibitors) at three different

sites (site II, III and IV) (Hudson and Daniel, 1993). These intricate catalytic and regulatory mechanisms have complicated identification of the putative acylated cysteine. Fortunately, bovine liver GluDH has only six cysteine residues (Amuro *et al.*, 1989).

1.6 Carbamoyl Phosphate Synthetase

Carbamoyl phosphate synthetase (CPS) catalyzes the formation of the first intermediate in arginine and urea biosynthesis, carbamoyl phosphate. The CPS family can be grouped into three major classes according to their requirement for the allosteric activator *N*-acetyl-L-glutamate (AGA) since it serves as a major regulatory feature of mammalian ammonia detoxification (Meijer *et al.*, 1990) and their nitrogen donor. The CPS 1 group require the allosteric activator AGA and utilize ammonia, not glutamine, as their nitrogen donor. Examples of this group are the rat liver and human liver CPS. By contrast, members of the CPS II group do not require AGA and prefer glutamine as their nitrogen donor (but can also utilize ammonia). Examples of this group are the arginine-specific and pyrimidine-specific CPSs (CPS II located in the mammalian cytosol liver and most other cells catalyzes the formation of carbamoyl phosphate destined for the synthesis of pyrimidine nucleotides) and the *E. coli* CPS of both pathways. CPS III, like the CPS I, group members requires AGA but, like CPS II prefers glutamine (it to can also use ammonia). Belonging to this group are the urea osmotic elasmobranches and some vertebrates.

1.6.1 Carbamoyl Phosphate Synthetase 1

The CPS 1 reaction is the first and rate limiting step of the urea cycle (Ratner, 1973; Krebs *et al.*, 1973, Tatibana and Shigesada, 1976). While the mechanism of the CPS 1 reaction remains unclear (Elliott and Tipton, 1974a; Jones, 1976), a three-step mechanism has been postulated (Jones, 1965; Powers and Meister, 1978). Free Mg^{2+}, K^{+} and the allosteric activator, AGA have been shown to be absolutely required for enzymatic activity (Tatibana and Shigesada, 1976).

Step 1: $$MgATP + HCO_3^- \xrightarrow{Mg^{2+},\ K^+;\ AGA,\ CPS\ 1} MgADP + carboxyphosphate$$

Step 2: $$carboxyphosphate + NH_3 \longrightarrow Pi + carbamate$$

Step 3: $$carbamate + MgATP \longrightarrow MgADP + carbamoylphosphate$$

$$2MgATP + NH_3 + HCO_3^- \xrightarrow{Mg^{2+},\ K^+;\ AGA,\ CPS\ 1} 2MgADP + Pi + carbamoylphosphate$$

CPS 1 consists of a single 165 kDa polypeptide chain (Clarke, 1976; Lusty, 1978) composed of a 40 kDa N-terminal domain and a 120 kDa C-terminal domain (Nyunoya, *et al.*, 1985b). Although originally discovered to be a liver mitochondrial matrix protein, it has also been shown to interact with the inner mitochondrial membrane. It is also found in the mitochondria of the small intestine (Gamble and Lehninger, 1973; Clarke, 1976). The concentration of CPS 1 in the mitochondrial matrix is estimated at 0.4 to 1.5 mM (Raijman and

Ellen Jones, 1976; Meijer *et al.*, 1990) with one third to one half of the enzyme in its active form. Levels of CPS 1 are 10 times lower in the small intestine (Ryall *et al.*, 1985). CPS 1 is an abundant mitochondrial protein, it represents 15-26 % of mitochondrial matrix protein and approximately 4 % of total liver protein (Lusty, 1978; Raymond and Shore, 1981; Clarke, 1976). CPS 1 has been purified from human (Pierson and Brien, 1980), rat (Guthohrlein and Knappe, 1968; Clarke, 1976; Lusty, 1978), bovine (Elliott and Tipton, 1973) and frog (Marshall *et al.*, 1958) liver.

1.6.2 Structure of CPS 1

Sequencing studies (Simmer *et al.*, 1990; Hong *et al.*, 1994; Lusty *et al.*, 1983; Nyunoya *et al.*, 1985a) have shown that CPS I, II and III share sequence identity and homology. Also, all CPSs incorporate a glutamine amidotransferase domain (GAT). Amidotransferases catalyze glutamine hydrolysis to supply ammonia for *de novo* biosynthesis of purines, pyrimidines, glucosamine, folate, nicotinamide, and several amino acids (Zalkin, 1990; Weng and Zalkin, 1987). With the exception of the mitochondrial CPS 1, all known carbamoyl phosphate synthetases use glutamine as the nitrogen donor (Marshall *et al.*, 1958). CPS 1 contains the GAT homology region but it cannot utilize glutamine as a substrate and it has no detectable glutaminase activity (Kerson and Appel, 1968) (Fig. 1.4). An essential cysteine required for glutaminase activity is replaced by a serine (294) in rat liver CPS 1 (Rubino *et al.*, 1986).

CPS 1 is synthesized with a leader sequence (residues 1-38/39). The leader sequence is cleaved upon entry of the 165 kDa precursor into the mitochondrion (Raymond and Shore, 1981) at residue 38 or 39 (Nyunoya, *et al.*, 1985a). Therefore, the N-terminal boundary of the protein is residue 39 or 40.

Limited proteolysis has revealed that CPS 1 is composed of four independently folded domains of approximately 40 (Domain A=residues 39-417), 40 (Domain B=residues 418-787), 60 (Domain C=residues 788-1328) and 20 (Domain D=1329-1500) kDa (Powers-Lee and Corina, 1986, 1987; Evans and Balon, 1988; Marshall and Fahien, 1988). The C/D domain interface is not well defined. V8 protease cleaved the C/D interface to include residue 1328 while trypsin and elastase cleaved the interface at residue 1356 (Marshall and Fahien, 1988).

1.6.3 Structural Features of CPS 1

1.6.3.1 AGA Binding

AGA is the allosteric activator of CPS 1 (Fahien *et al.*, 1964; Marshall *et al.*, 1961; Tatibana and Shigesada, 1976). The intramitochondrial concentration of AGA was estimated to be 2-4 x 10^{-4} M (Shigesada and Tatibana, 1971) and this increases with protein load in the diet (Shigesada and Tatibana, 1971; Stewart and Walser, 1980). Alonso and Rubio (1983) demonstrated that one molecule of AGA binds one molecule of the CPS 1 enzyme (mass 160 kDa) reversibly and noncovalently. AGA does not participate directly in the CPS 1 reaction (Rubio *et al.*, 1983), rather it activates CPS 1 greater than 50 fold by inducing conformational changes (Rubio *et al.*,

1983) which ultimately expose essential thiol (SH) groups within CPS 1 (Novoa *et al.*, 1966; Marshall and Fahien, 1985) and its ATP-binding sites (Rubio *et al.*, 1983). Hence, this activation of CPS 1 leaves it very sensitive to thermal (Caravara and Grisolia, 1959; Fahien *et al.*, 1964), oxidative (Alonso and Rubio, 1987) and proteolytic (Guadlajara *et al.*, 1987; Marshall and Fahien, 1988) inactivation. McCudden and Powers-Lee (1996) labeled CPS 1 with [^{14}C]AGA and identified peptides 120-127, 234-237, 625-630 and 1351-1356 as potentially being close to the binding site for AGA (Fig. 1.4). Identification of peptide 1351-1356 confirmed previous work by Rodriguez-Aparicio *et al.*, (1989) that the C-terminal region of CPS 1 is involved in AGA binding. Identification of peptides 120-127 and 234-237 was the first evidence demonstrating that the N-terminal of CPS 1 is involved in ligand binding.

1.6.3.2 ATP Binding

Two molecules of ATP are used in the reaction catalyzed by CPS 1 (Marshall, 1976), one molecule (ATP_B for Step 1) for the activation of bicarbonate and the other (ATP_C for Step 2) for the phosphorylation of enzyme-bound carbamate (Ratner, 1973; Rubio *et al.*, 1983) (Fig. 1.4). Kinetic and pulse-chase studies have suggested that there are two separate ATP sites on CPS 1 (Rubio *et al.*, 1979; Britton *et al.*, 1979). There is no direct evidence available on the residues that are involved in binding the two ATP molecules. Sequence analysis of CPS 1 revealed internal duplication and comparison with other nucleotide binding proteins, identified potential ATP sites in each of the these duplicated sequences (Nyunoya *et al.*, 1985a; Powers-Lee and Corina,

1987). Therefore, domain B was identified as the core locus for the ATP_B site and domain C was identified as the core locus for the ATP_C site. In addition, further studies with the ATP affinity labels 8-azido-ATP and 5'-p-fluorosulfonylbenzoyladenosine (FSBA) identified that 2 mol of the analog/mol of enzyme inactivate the enzyme (Powers-Lee *et al.*, 1983; Powers-Lee and Corina, 1987) again suggesting the presence of two distinct MgATP sites, but investigation into defining the sites was yet to be performed.

In 1992, Potter and Powers-Lee used the ATP analog, 5'-p-fluorosulfonylbenzoyladenosine (FSBA), to identify peptides 631-637, 1310-1317, 1327-1348 and 1445-1454 as the ATP binding domains (Fig. 1.4). The identifications of peptides 631-637 and 1310-1317 were consistent with previously reported sequence analyses of MgATP-binding sites (Nyunoya *et al.*, 1985a; Powers-Lee and Corina, 1987). This was the first demonstration that peptides 1327-1348 and 1445-1454 in the extreme C-terminus of the protein were involved in binding MgATP (Potter and Powers-Lee, 1992). The requirement for peptides 1327-1348 and 1445-1454 for binding MgATP would explain the total loss of CPS 1 enzymatic activity observed during C-terminal proteolysis (Powers-Lee and Corina, 1986; Marshall and Fahien, 1988; Evans and Balon, 1988). An increase in the affinity of the enzyme for ATP_B, was observed in the presence of the allosteric activator of CPS 1, AGA, but the binding affinity of ATP_C to the CPS 1 was similar whether or not acetylglutamate was present (Rubio *et al.*, 1983; Britton and Rubio, 1988). Potter and Power-Lee (1992) observed a decrease in labeling of peptides 631-638 and 1327-

1348 with FSBA when AGA was present suggesting that these peptides are involved in the binding of MgATP required for bicarbonate activation (ATP_B) and peptides 1310-1317 and 1445-1454 are involved in the binding site for MgATP involved in phosphorylation of enzyme-bound carbamate (ATP_C).

Interestingly, two peptides identified as part of the potential AGA binding domain (625-630 and 1351-1356) (McCudden and Powers-Lee, 1996) are next to those peptides identified as the ATP_B binding site (631-638 and 1327-1348) (Potter and Powers-Lee, 1992) suggesting that the required allosteric activator AGA binds to a site that is very near the active site of CPS 1 and facilitates ATP_B binding.

1.6.3.3 Cysteine Residues

CPS 1 contains 21 cysteine residues which are distributed throughout the protein, none of which are involved in disulphide linkages (Clarke, 1976; Powers, 1981) (Fig. 1.4).

1.6.3.4 Regulation

CPS 1 is coded for by a single copy nuclear gene (Nyunoya *et al.*, 1985a; Adcock and O'Brien, 1984). It has been demonstrated that CPS 1 enzyme levels decrease with protein-free diets and increase with high protein diets, starvation or administration of glucagon (leading to increased intracellular cAMP) or glucocorticosteroids (Schimke, 1962a; Schimke, 1962b; Nuzum and Snodgrass, 1971; Tsuda *et al.*, 1979; Schimke, 1963; Snodgrass *et al.*, 1978). Increase in formation of AGA, the required allosteric activator of CPS 1 is also observed with amino acid load (Powers, 1981). CPS 1 is also rapidly activated

by thiols, ammonium salts or ornithine *in vivo* (Powers, 1981; Krebs *et al.*, 1973; Tatibana and Shigesada, 1976; Saheki and Katsunuma, 1975). Carbamoyl phosphate production is increased by the substrate ammonia, since the K_m of CPS 1 for NH_4^+ is 0.4-2 mM (Guthohrlein and Knappe, 1968; Raijman and Jones, 1976; Lusty, 1978; Pierson and Brien, 1980; Elliott and Tipton, 1974a; Elliott and Tipton, 1974b) and the average concentration of NH_4^+ in the liver is 0.5 mM (Ratner, 1973). However, CPS 1 activation by ornithine remains unelucidated. Ornithine may serve to increase the arginine concentration, resulting in increased synthesis of AGA (Krebs et al., 1973). CPS 1 is also inhibited by physiological levels of heavy metal ions *in vitro* but this inhibition is reversed by ornithine and some other amino acids due to their ability to chelate heavy metal ions (Powers, 1981). Interestingly, rat primary hepatocyte CPS 1 mRNA levels induced by the glucocoricoid hormone, dexamethasone was suppressed by saturated long chain fatty acids of more than 16 carbons in chain length (Tomomura *et al.*, 1996). This effect was also enhanced by the presence of double bonds in long chain fatty acids (Tomomura *et al.*, 1996).

1.6.4 Model for CPS 1

The crystal structure of CPS 1 has yet to be solved but McCudden and Powers-Lee (1996) have proposed a model for CPS 1 based on the AGA, ATP and limited proteolysis findings (Fig. 1.5). They propose that the peptides identified in the AGA binding studies act at a single binding site (120-127 and 234-237 located in domain A, 625-630 located in domain B, and 1351-1356 located in the domain C/D interface), therefore allowing interaction between

domains A and D. In support of this, secondary structure analysis techniques have predicted the C-terminal of CPS 1 to be very flexible due to its many β-turns, random coil areas, and short stretches of α-helix or β-sheet structures (Chou and Fasman, 1978; Garnier *et al.*, 1978). This would allow for the C-terminal region or domain D to fold back over and interact with both large internal domains B and C of the enzyme which are proposed to contain the two ATP binding sites. This interaction would form close sites for the binding of ATP_B, AGA and ATP_C. Also in support of this model is an ATP data model proposed by Potter and Powers-Lee (1993), where domain A was shown to interact with domain B and/or C and that domain B participates in binding the molecule of ATP involved in bicarbonate activation and domain C participates in binding the molecule of ATP involved in carbamate phosphorylation.

1.6.5 Metabolic Disorders Relating to CPS 1 Deficiency

A variety of diseases have been associated with deficiencies in CPS 1 including methylmalonic aciduria (Nicoletti *et al.*, 1977), migraine syndrome (Russell, 1969), Reye's syndrome (Thaler *et al.*, 1974; Brown *et al.*, 1974; Sinatra *et al.*, 1975; Snodgrass and Delong, 1976; Brown *et al.*, 1976), ornithine transcarbamylase deficiency (Palmer *et al.*, 1974), and cogenital hyperammonemia (Snodgrass and Delong, 1976; Batshaw *et al.*, 1975).

1.7 Objectives of Research

While relatively little is known about the fatty acylation of proteins in the mitochondria, other fatty acylated proteins (between 6-10) have been observed in rat liver and COS-7 mitochondria (Stucki *et al.*, 1989; Berthiaume *et al.*, 1994)

but the identity of these proteins is unknown as is their potential role of their fatty acylation in the regulation of mitochondrial metabolism. Prior to this work, only one fatty acylated protein has been identified, MMSDH (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). MMSDH, a mitochondrial enzyme involved in the valine and pyrimidine catabolic pathways, was fatty acylated on its active site cysteine residue (Cys319) thereby inhibiting its enzymatic activity (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). A model was proposed in which MMSDH's fatty acylation could act as novel mode of regulation of its enzymatic activity within the mitochondria, suggesting the possibility of a metabolic cross-talk between amino acid and fatty acid catabolic pathways (Berthiaume *et al.*, 1994). But the model was proposed and supported by only one protein. Berthiaume and coworkers (1994) also demonstrated that bovine liver GluDH bound fatty acid covalently but the chemical nature of the palmitate bond or location of the potential palmitoylation site were not investigated.

The objective of the work presented in this thesis was to further explore and characterize mitochondrial protein fatty acylation and also to determine if protein fatty acylation is a general mechanism of metabolic regulation in the mitochondria. In order to do so, we need look at fatty acylation of proteins in different tissues and also to characterize the role of protein fatty acylation in newly purified fatty acylated proteins. Therefore, the goals of my work were, (1) to characterize mitochondrial protein fatty acylation by looking at protein fatty acylation patterns in mitochondria isolated from different tissues; (2) to purify and identify new fatty acylated proteins from rat liver mitochondria; and (3) to

further characterize the function of their fatty acylation in regard to enzyme activity.

Table 1.1 Characteristics of the major classes of lipidated proteins.

Shown are the four major classes of lipidation and their subclasses. The structure of the lipid and the attachment residue are shown, except for the complex GPI moiety. The subunit to which the lipid is attached is indicated in parentheses after the trimeric G protein. NRTKs- nonreceptor tyrosine kinases, GPCRs-G protein-coupled receptors, Retinal GRK-specific receptor kinases (Adapted from Casey, 1995 and Bhatnagar and Gordon, 1997).

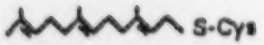
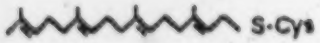
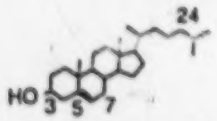
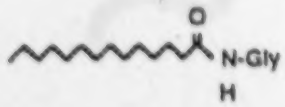
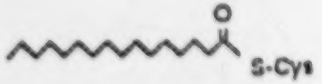
Modification	Structure	Representative proteins modified	Position of Modification	Chemistry
Prenylation				
Farnesylation		Trimeric G Proteins (γ), Small G proteins, Retinal GRK	C-terminus	Thioether; stable
Geranyl-geranylation				
Glypiation	Complex structure containing ethanolamine, sugars, and phosphatidylinositol	Many cell-surface proteins eg. parasite protazoan Trypanosoma brucei (causative agent of African sleeping disease)	C-terminus	Amide; stable
Cholesteroylation		Hedgehog (Hh) family of secreted signaling proteins	C-terminus	Ester; labile
Fatty acylation				
Myristoylation		Trimeric G proteins (α), NRTKs	N-terminus	Amide; stable
Palmitoylation		Trimeric G proteins (α), GPCRs, Ras, MMSDH	Internal, no defined consensus	Thioester; labile

Table 1.2 Characteristics of the two categories of fatty ayclation: myristoylation and palmitoylaton.

NMT- *N*- myristoyltransferase, PAT- protein acyl transferase

	Myristoylation	Palmitoylation
Fatty Acid	Myristic (C14:0)	Palmitic (C16:0)
Type of linkage	Amide	Thioester
Modified residues	N-terminal Gly	Cys
Reversibility	Irreversible	Reversible
Enzymology	NMT well characterized	PAT unknown
Consensus Sequence	MGXXX(C/S/T)XX	None apparent

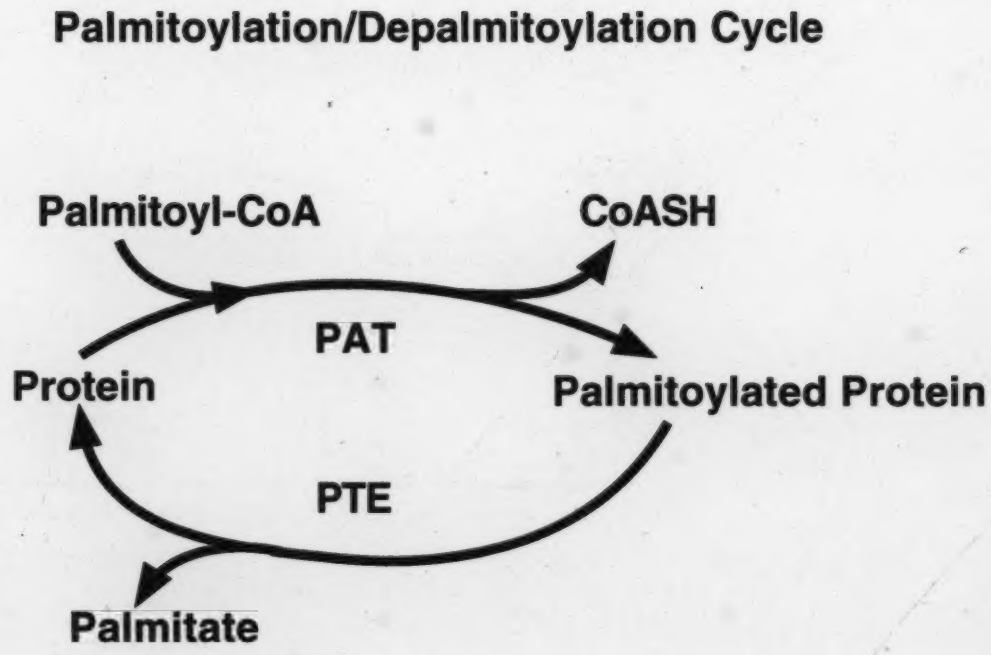
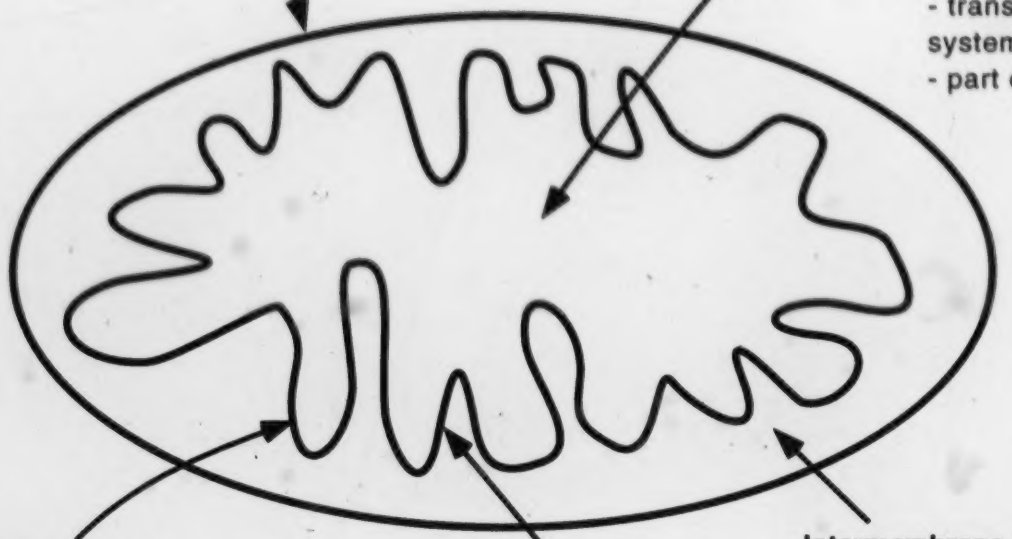


Figure 1.1 Model describing the regulation of reversible protein palmitoylation.

In the majority of cases the reversibility of protein palmitoylation is thought to be governed by the actions of protein acyltransferases (PAT) and protein thioesterases (PTE). Palmitoylation may occur at one or more sites on the protein. As illustrated above, the protein becomes palmitoylated by PAT to form a palmitoylated protein.

Figure 1.2 A schematic diagram of the four components of a mitochondrion.

The principal enzymes and pathways in each compartment are shown (Adapted from Moran *et al.*, 1994).



Outer membrane
- receptor proteins
- protein import system

Matrix
- TCA cycle
- pyruvate dehydrogenase complex
- heme biosynthesis
- mtDNA replication
- transcription/translation system
- part of the urea cycle

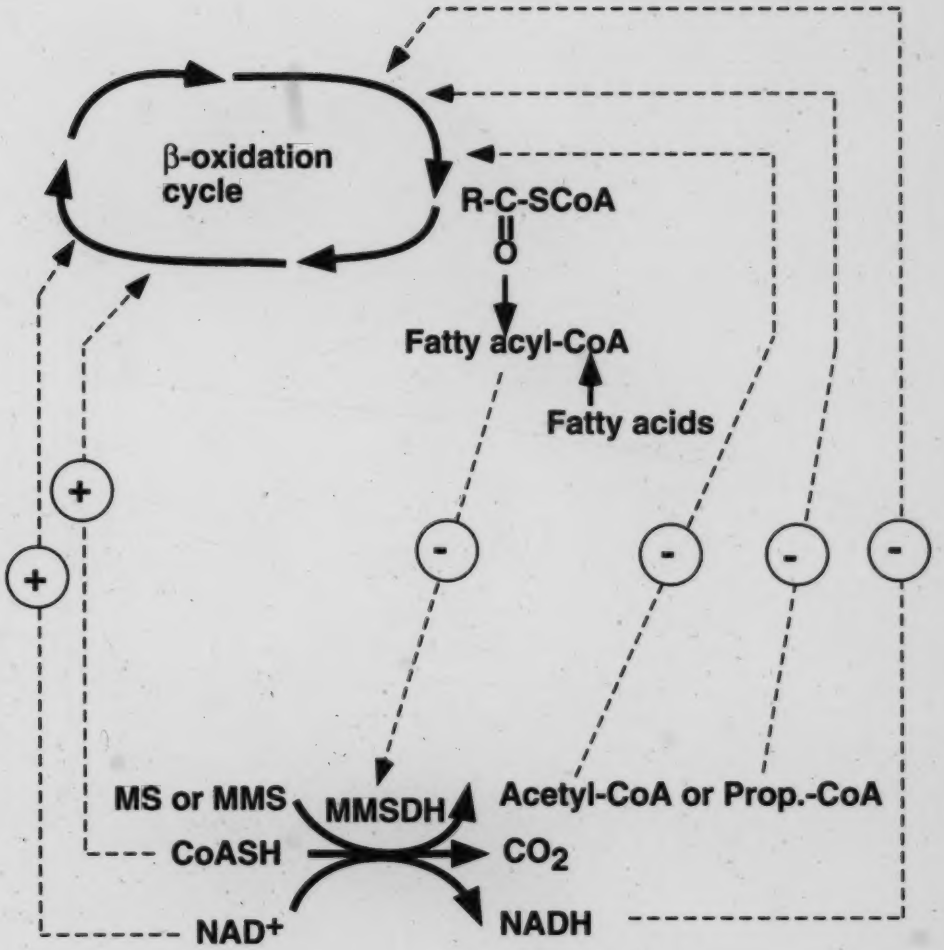
Inner membrane
- Electron Transport Chain (ETC)
- ATP synthase
- protein import system
- ATP/ADP transport system

Cristae

Intermembrane Space
- cytochrome c
- lactate dehydrogenase

Fig. 1.3 A model for the regulation of MMSDH by active site fatty acylation.

MMSDH competes with β-oxidation for the cofactors, CoASH and NAD^+, which have a positive effect on β-oxidation. The products of the MMSDH reaction, acetyl-CoA and propionyl-CoA and the cofactor NADH, all have inhibitory effects on β-oxidation. Therefore, when MMSDH is active, it has a negative effect on β-oxidation. But when MMSDH becomes fatty acylated by long-chain fatty acyl-CoAs, its acitivty is inhibited releving the negative effect on β-oxidation. (Adapted from Berthiaume *et al.*, 1994)


β-oxidation cycle
R-C-SCoA
O
Fatty acyl-CoA
Fatty acids
+
+
-
-
-
-
MS or MMS
MMSDH
Acetyl-CoA or Prop.-CoA
CoASH
CO2
NAD+
NADH

Amino Acid Number

```
MTRILTACKVVKTLKSGFGLANVTSKRQMDFSRPGIRLLSVKAQTAHIVLEDGTKMKGYSFGHPSSVAGE      70
VVFNTGLGGYSEALTDPAYKGQILTMANPIIGNGGAPDTTARDELGLNKYMESDGIKVAGLLVLNYSHDY     140
NHWLATKSLGQWLQEEKVPAIYGVDTRMLTKIIRDKGTMLGKIEFEGQSVDFVDPNKQNLIAEVSTKDVK     210
VFGKGNPTKVVAVDCGIKNNVIRLLVKRGAEVHLVPWNHDFTQMDYDGLLIAGGPGNPALAQPLIQNVKK     280
ILESDRKEPLFGISTGNIITGLAAGAKSYKMSMANRGQNQPVLNITNRQAFITAQNHGYALDNTLPAGWK     350
PLFVNVNDQTNEGIMHESKPFFAVQFHPEVSPGPTDTEYLFDSFFSLIKKGKGTTITSVLPKPALVASRV     420
EVSKVLILGSGGLSIGQAGEFDYSGSQAVKAMKEENVKTVLMNPNIASVQTNEVGLKQADAVYFLPITPQ     490
FVTEVIKAERPDGLILGMGGQTALNCGVELFKRGVLKEYGVKVLGTSVESIMATEDRQLFSDKLNEINEK     560
IAPSFAVESMEDALKAADTIGYPVMIRSAYALGGLGSGICPNKETLMDLGTKAFAMTNQILVERSVTGWK     630
EIEYEVVRDADDNCVTVCNMENVDAMGVHTGDSVVVAPAQTLSNAEFQMLRRTSINVVRHLGIVGECNIQ     700
FALHPTSMEYCIIEVNARLSRSSALASKATGYPLAFIAAKIALGIPLPEIKNVVSGKTSACFEPSLDYMV     770
TKIPRWDLDRFHGTSSRIGSSMKSVGEVMAIGRTFEESFQKALRMCHPSVDGFTPRLPMNKEWPANLDLR     840
KELSEPSSTRIYAIAKALENNMSLDEIVKLTSIDKWFLYKMRDILNMEKTLKGLNSESVTEETLRQAKEI     910
GFSDKQISKCLGLTEAQTRELRLKENIHPWVKQIDTLAAEYPSVTNYLYVTYNGQEHDIKFDEHGIMVLG     980
CGPYHIGSSVEFDWCAVSSIRTLRQLGKKTVVVNCNPETVSTDFDECDKLYFEELSLERILDIYHQEACN    1050
GCIISVGGQIPNNLAVPLYKNGVKIMGTSPLQIDRAEDRSIFSAVLDELKVAQAPWKAVNTLNEALEFAN    1120
SVGIPCLLRPSIVLSGSAMNVVFSEDEMKRFLEEATRVSQEHPVVLTKFIEGAREVEMDAVGKEGRVISH    1190
AISEHVEDAGVHSGDATLMLPTQTISQGAIEKVKDATRKIAKAFAISGPFNVQFLVKGNDVLVIECNLRA    1260
SRSFPFVSKTLGVDFIDVATKVMIGESVDEKHLPTLEQPIIPSDYVAIKAPMFSWPRLRDADPILRCEMA    1330
STGEVACFGEGIHTAFLKAMLSTGFKIPQKGILIGIQQSFRPRFLGVAEQLHNEGFKLFATEATSDWLNA    1400
NNVPATPVAMPSQEGQNPSLSSIRKLIRDGSIDLVINLPNNNTKFVHDNYVIRRTAVDSGIALLTNFQVT    1470
KLFAEAVQKARTVDSKSLFHYRQYSAGKAA                                              1500
```

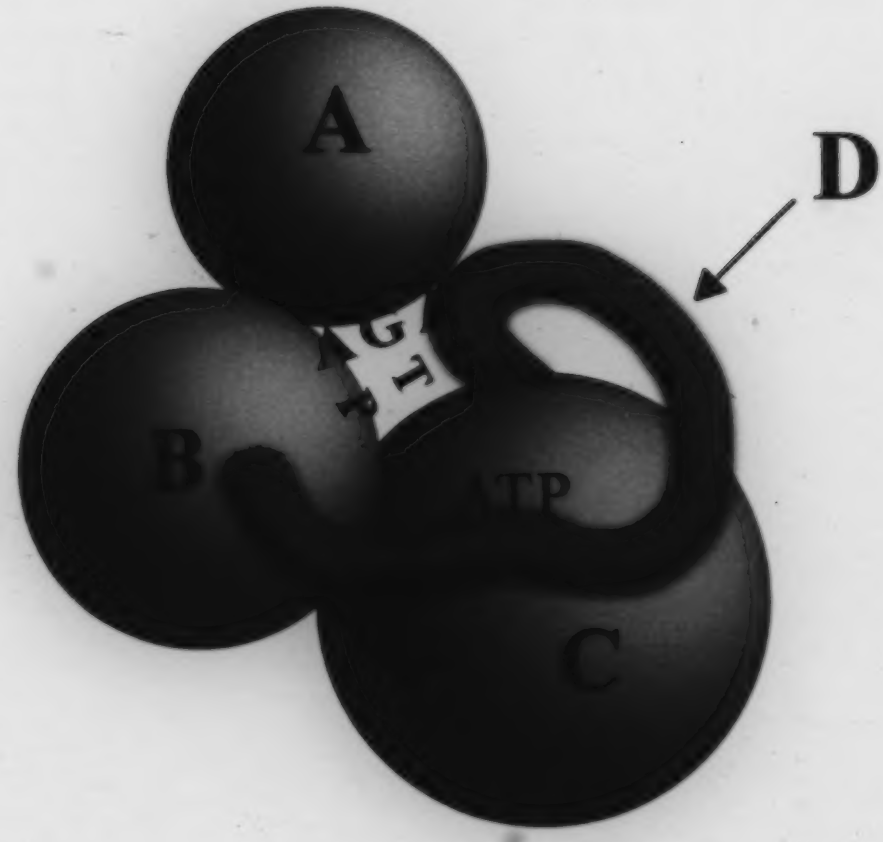
Fig. 1.4 Rat liver Carbamoyl Phosphate Synthetase 1 (CPS 1) Amino Acid Sequence

The complete amino acid sequence of rat liver CPS 1 is shown. The encoded protein is composed of 1500 amino acids and has a predicted molecular mass of 165 kDa (Adapted from Nyunoya *et al.*, 1985a and Lagace *et al.*, 1987).

LEGEND
MITOCHONDRIAL LEADER PEPTIDE (1-39)
GLUTAMINE AMIDOTRANSFERASE-LIKE DOMAIN (220-410)
ATP BINDING SITE 1 (631-637,1327-1348 BY ATP CROSS-LINKING)
ATP BINDING SITE 2 (1310-1317,1445-1454 BY ATP CROSS-LINKING)
N-ACETYLGLUTAMATE BINDING SITES (120-127,234-237,625-630, 1351-1356)
C CYSTEINE RESIDUES
CARBAMYL PHOSPHATE SYNTHETASE LARGE CHAIN FOLLOWS THE GLUTAMINE AMIDOTRANSFERASE-LIKE DOMAIN (411-1500)

Fig. 1.5 Schematic of the CPS 1 structure.

CPS 1 is composed of four independently folded domains where domain A consists of residues 39-417, domain B consists of residues 418-787, domain C consists of residues 788-1328 and domain D consists of residues 1329-1500. The AGA binding site contains parts from both domains A and D. Domain B is close to domains A and D and may also pariticipate in the AGA site. The ATP_B site is on domain B with overlapping components from domain D. The ATP_C site is on domain C, also with overlapping components from domain D (Adapted from McCudden and Powers-Lee, 1992).


A
D
B
C

2.0 MATERIALS AND METHODS

2.1 Materials

Cell lines, growth media, antibiotics.

McArdle RH7777 (McA) cells were obtained from the American Type Tissue Culture Collection (ATCC) (Rockville, MD, USA). Culture media, Fetal Bovine Serum (FBS), Horse Serum (HS), trypsin-EDTA (0.25 % trypsin (w/v), 1 mM ethylenediaminetetraacetate), penicillin and streptomycin were obtained from Gibco BRL.

Chemicals and reagents.

Reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from Gibco BRL. Bovine serum albumin (BSA), fatty acyl-CoAs, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), N-ethyl maleimide (NEM), pyruvate kinase, and lactate dehydrogenase were from Sigma. Leupeptin and bovine liver glutamate dehydrogenase were from Boehringer Mannheim. Mono Q HR 5/5 and Phenyl Superose HR 5/5 columns, and Hiprep Sephacryl-200 26/60, SP Sepharose HP and Phenyl Sepharose gels were from Amersham-Pharmacia Biotech Inc. (Baie d'Urfe, Quebec, Canada). Macro-Prep Ceramic hydroxyapatite (40 μ beads) were from Bio-Rad (Hercules, California, USA). Broad Range SDS PAGE protein markers were obtained from either New England Biolabs or Bio-Rad. Other materials were obtained from standard commercial sources.

Animals

Sprague-Dawley rats were a kind gift of Dr. G. Lopaschuk (University of Alberta). Balb/c mice were a kind gift of Dr. S. Yokoyama (University of Alberta).

Buffers and Solutions.

All solutions were prepared from analytical grade reagents in double distilled water.

Radioactive materials

[^{125}I]NaI was purchased from Amersham Pharmacia Life Science (Baie d'Urfe, Quebec, Canada).

2.2 Cell Culture

2.2.1 Media and Cell Culture Conditions

Frozen stocks of cells were prepared by combining trypsinized cells with freezing medium consisting of 10 % DMSO, 20 % FBS and 10 % DMEM. Aliquots of the cells to be frozen were then place in cryovials (Nalge), placed on ice for 15 minutes, transferred to -70 °C overnight, and then transferred to liquid nitrogen for long term storage. Frozen stocks were thawed rapidly in a beaker of MilliQ water (25 °C) and transferred to 12 ml of medium (10% FBS, 10% DMEM) in a 100 mm dish. After incubating overnight (37 °C, 5 % CO_2) the medium was removed and replaced with fresh medium.

The McA RH7777 cell line was cultured in DMEM containing 10 % FBS (v/v), 10 % heat-inactivated HS (v/v), 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate. Cells were passed twice weekly when 80 % confluent using a 0.25 % trypsin/1 mM EDTA wash. The cells were grown at 37

°C in a humidified atmosphere of 5 % CO_2. Cells were plated at a density of 1 x 10^6 cells/100 mm dish as determined by counting with a hemacytometer. When growth reached 70% confluence, mitochondria were prepared as described below. The cell culture line was maintained by Yang Zhao, technician to Dr. L. Berthiaume (University of Alberta).

2.2.2 Preparation and Culture of Hepatocytes

Rat primary hepatocytes used in this study were isolated from the livers of Sprague-Dawley rats by the previously described collagenase perfusion technique (Davis *et al.*, 1979; Vance *et al.*, 1984). Briefly, rats were anesthetized with ether followed by injection with sodium pentobarbital (0.022 ml/50g rat). The portal vein was cannulated and perfused according to Davis *et al.*, (1979). After perfusion, the liver was excised and transferred to a tube containing 15 ml of Hanks-collagenase solution. The tube was shaken in a 37 °C water bath for 1.5 minutes and the collagenase reaction was stopped by the addition of DMEM containing 17 % FBS and 0.8 mg of insulin. The cells were filtered through a coarse filter and cells were pelleted at 500 x *g* in a clinical centrifuge, two times 5 minutes, and the supernatant was discarded. This was repeated 3 times, then the cells were filtered through a fine filter and DMEM was added to a volume of 100 ml. The cells were plated at a density of 3 x 10^6/60-mm in Eagle's minimum essential medium containing 17 % FBS and were allowed to adhere to the dishes for 16 hours at 37 °C in an atmosphere of 5 % CO_2. Rat primary hepatocytes were prepared by Russ Watts, technician to Dr. J.

Vance (University of Alberta). Mitochondria were prepared the following day as described below.

2.3 Preparation of Mitochondria from tissues

Mitochondria were purified from rat liver, heart, brain, kidney and leg muscle from 5 Sprague-Dawley rats (approximately 200 g individually) or from 10 Balb/c mice, using a combination of differential and Percoll gradient centrifugation as previously described (Vance, 1990). All buffers contained freshly added 1 mM DTT, 20 μg/ml leupeptin and 1 mM PMSF.

Tissues were removed and transferred to 2 volumes of homogenization buffer (20 mM Tris-HCl pH 7.85, 10 % sucrose, 0.1 M PMSF and 1 mM DTT). After rinsing the tissues in homogenization buffer, the fresh tissue was removed from the buffer and minced into fine squares on ice, then homogenized in 4 times the weight of the tissue in Isolation Medium (IM: 250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EDTA, 0.1 % albumin (essentially fatty acid free)) in a Potter-Elvehjem glass tube. Rat heart and leg muscle were also minced prior to mitochondrial isolation with a Polytron at level 2.5 for 30 seconds. In the cases where mitochondria were not prepared immediately, the tissues were minced, immersed in homogenization buffer containing 20 % DMSO, and incubated on ice for 20 minutes before freezing at -80 °C.

After homogenization, unbroken cells and nuclei were pelleted by centrifugation (600 x *g*, 5 min x 2). The supernatant, containing the mitochondria, was centrifuged at 10 300 x *g* for 10 minutes at 4 °C to pellet the mitochondria, the resulting supernatant was discarded. Isolated mitochondria

were gently resuspended in a minimal volume of IM. Mitochondria (1 ml/tube) were layered onto a 30 % Percoll solution (PIM: 225 mM mannitol, 25 mM HEPES, 1 mM EGTA, 0.1 % BSA albumin (essentially fatty acid free), 30 % Percoll (v/v)) (ratio mitochondria:Percoll=1:20) and centrifuged at 95 000 x *g* in a Ti 70 rotor for 30 minutes at 4 °C. The brown mitochondrial band was collected by aspiration using a glass Pasteur pipette. The collected mitochondria were pooled, diluted 20 fold with IM, pelleted by centrifugation at 6 300 x *g* for 10 minutes at 4 °C in a JA 20 rotor, washed again and then pelleted as before. The resulting supernatant was removed and the mitochondria were resuspended in 0.5 ml of IM containing 20 μg/ml leupeptin (Powers-Lee and Corina, 1986). The mitochondria were homogenized with 5 gentle strokes of a loose glass homogenizer, aliquoted into 1.5 ml eppendorf tubes and frozen at -80 °C. When aliquots were required, they were removed from -80 °C and placed on ice until thawed. The aliquots were never thawed and refrozen.

2.4 Preparation of Mitochondria from McArdle RH7777 Cells and Rat Primary Hepatocytes

Mitochondria were purified using the procedure of Bogenhagen and Clayton (1974) as modified by Berthiaume and coworkers (1994). Mitochondria were prepared from cells corresponding to 6-10 70 % confluent 100 mm plates. Cells were rinsed twice with cold STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4) (Resh and Erickson, 1985). Five ml of cold 1 X TD buffer (134 mM NaCl, 3 mM KCl, 0.7 mM Na_2PO_4, 2.5 mM Tris pH 7.5) was added to each plate, the cells were manually scraped from the plates with a rubber policeman (Resh and

Erickson, 1985) and collected in a 50 ml Falcon tube. The isolated cells (~30 ml) were pelleted by centrifugation for 5 minutes at 1000 rpm in a clinical centrifuge (25 °C). The supernatant was discarded and the pellet was resuspended in 30 ml of 1 X TD buffer. The cells were pelleted again, the supernatant discarded and 30 ml of 1 X MgRSB solution (10 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris pH 7.4) was added to resuspend the cells. The cells were allowed to swell on ice for 10 minutes. The cells were then dounced 10 times with a "tight" glass pestle, 20 ml of 2.5 X MS solution (0.525 M mannitol, 0.175 M sucrose, 12.5 mM Tris pH 7.5, 12.5 mM EDTA) was added and dounced twice more. The cell debris was pelleted two times for five minutes at 2500 rpm in a clinical centrifuge (25 °C) and discarded. The combined supernatant was centrifuged at 12 000 x *g* in a JA 20 rotor for 20 minutes (4 °C). The supernatant was removed and the mitochondrial pellet was washed delicately twice with 10 ml of 1 X MS buffer. The mitochondria were centrifuged at 12 000 x *g* in a JA 20 rotor for 5 minutes and the mitochondrial pellet was resuspended in 1 ml of 1 X MS, dounced 3 times with a "loose" glass pestle and frozen at -80 °C in aliquots. The aliquots were never thawed and refrozen.

2.5 Subfractionation of Rat Liver Mitochondria

Mitochondria were prepared from two rat livers as described above and were subfractionated by the swell-shrink-sonication procedure according to Ohlendieck *et al.*, (1986) followed by discontinuous density gradient centrifugation according to Parsons and Williams, (1967).

The final mitochondrial pellet obtained from the 2 rat livers was resuspended in swell medium (10 mM KPi, pH 7.4, 100 mM PMSF, 1 mM DTT) to a total volume of 6 ml and incubated while stirring for 15 minutes at 4 °C. After 15 minutes, 6 ml of shrink medium (10 mM KPi, 10 mM $MgCl_2$, 30 % glycerol) was added and the mixture was incubated for another 15 minutes while stirring on ice. The mixture was then sonicated twice for 15 seconds with a 1 minute rest in-between with a Brandson B-12 sonicator equipped with a 5 mm tip at a power output of 60 to 70 Watts. After sonication, the mixture was centrifuged for 10 minutes at 12 000 x *g*. at 4 °C. The pellet and supernatant were separated and the pellet was resuspended in approximately 8 ml of IM. The supernatant and resuspended pellet were loaded onto separate discontinuous sucrose gradient medium (25.3 %/ 37.3 %/ 51.3%/ sample at 1:1:1:2 ratio) and centrifuged for 3 hours at 170 000 x *g* (40 000 rpm) in a Beckman SW 40 Tl rotor at 4 °C. The outer membrane fraction (OM) was collected from the 37.7%/25.3% interface of the supernatant spin and was further centrifuged for 1 hour at 255 000 x *g* (58 000 rpm) in a 70 Ti rotor at 4 °C. The Intermembrane mitochondrial space (IMS) was removed from the same tube at the 51.3%/37.7% interface and washed with IM. From the pellet spin, the inner membrane (IM) fraction from the 51.3%/37.7% interface and the pelleted matrix (M) fraction were removed and centrifuged further for 10 minutes at 12 000 x *g* in a SS 34 rotor. After isolation, all samples were resuspended in IM and either used immediately or frozen at -20 °C with 20 μg/μl leupeptin. Protein concentration was determined by the Lowry assay (Lowry *et al.*, 1951)

using BSA as a standard. Marker enzymes for submitochondrial fractions were used to determine the purity of the fractions as follows. Citrate synthase (matrix) was assayed by the procedure of Srere (1969). Succinate-cytochrome c reductase (inner membrane) was assayed by the procedure of Taylor *et al.*, (1993). NADH cytochrome c reductase (outer membrane) was measured according to Sotocasa *et al.*, (1967). Submitochondrial compartments, protein concentrations and purity assays were prepared and performed by Benedicte Balcerzak, post-doctorate to Dr. J. Vance (University of Alberta).

2.6 Preparation of Radiolabeled Fatty Acids

In this study, the analogs of palmitate and palmitoyl-CoA: [^{125}I]iodopalmitate and [^{125}I]iodopalmitoyl-CoA were used to identify mitochondrial palmitoylated (fatty acylated) proteins (Peseckis *et al.*, 1993; Berthiaume *et al.*, 1995).

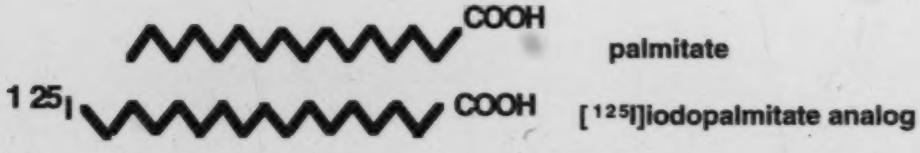


Fig 2.1 Structures of palmitate and the [^{125}I]iodopalmitate analogs.

Long chain fatty acids (>10 carbons) are impermeable to intact mitochondrial membranes, therefore, when labeling whole mitochondria, the mitochondria are incubated with the [^{125}I]iodopalmitate analog in a cocktail containing all of the necessary cofactors (ATP, CoA, Mg^{2+}, carnitine) to facilitate

mitochondrial fatty acid import (Stucki *et al.*, 1989; Berthiaume *et al.*, 1994). Malate, glutamate and rotenone (a respiratory chain inhibitor) are also added because a reducing environment is also required inside the mitochondrial matrix (high $NADH/NAD^+$) to inhibit fatty acid β-oxidation. When labeling mitochondrial protein fractions, protein fractions are labeled with the [^{125}I]iodopalmitoyl-CoA analog, since the covalent bond formation between proteins and fatty acids often requires activation of the latter in the form of the CoA derivative. Radiolabel incorporation into proteins after electrophoresis and autoradiography is taken as evidence that these proteins have been fatty acylated by the [^{125}I]iodopalmitate or [^{125}I]iodopalmitoyl-CoA analog.

2.6.1 Preparation of the iodopalmitate analog

Radioiodination of the iodopalmitate analog with [^{125}I]NaI (2.14 Ci/mmole) was performed according to Berthiaume and coworkers, (1995) without the HPLC purification step. 250 µl of acetone was mixed with 5 mCi of [^{125}I]NaI in a reaction vial containing 2 µmol of IC16 that was previously treated with 2 µl glacial acetic acid. The reaction vial was sealed and placed in a 55 °C heat block for 16 hours. 0.5 ml of dH_20 was added and the aqueous layer was extracted two times with 1.2 ml of chloroform. The combined chloroform layers were transferred to a borosilicate glass vial and reduced to dryness with a nitrogen stream. 1070 µl of 95 % EtOH was added to the vial and the IC16 was then aliquoted into 10 vials. The vials were and reduced to dryness with a nitrogen stream then stored at -20 °C. Typical specific activity of the [^{125}I]iodopalmitate obtained was approximately 2 Ci/mmole. The radioiodination

of the iodopalmitate (IC16) was prepared by Yang Zhao, technician to Dr. L. Berthiaume (University of Alberta).

2.6.2 Preparation of the iodopalmitoyl-CoA derivative

Synthesis of the [^{125}I]labeled IC16-CoA derivative was prepared using *Pseudomonas* acyl-CoA synthetase (Sigma) according to Berthiaume *et al.*, (1995). A borosilicate glass vial containing [^{125}I]iodopalmitate (~500 μCi/vial) was warmed to room temperature and 5 μl of 95 % EtOH was added while swirling. Next reaction buffer: (10 mM Tris-HCl pH 7.4, 1 mM DTT, 0.1 mM EGTA, 5 mM ATP, 5 mM $MgCl_2$ and 1 mM LiCoA) was added to the vial, followed by the addition of the acyl-CoA synthetase (300 milliunits/ml) to start the reaction. The vial was mixed gently and allowed to incubate at 30 °C for 30 minutes. The final concentration of the [^{125}I]iodopalmitoyl-CoA analog was approximately 1 μCi/μl and was used immediately or stored at -80 °C.

2.7 *In vitro* fatty acylation reactions

Highly purified mitochondria were incubated in the mitochondrial acylation buffer (MAB) (60 mM KCl, 7.5 mM potassium phosphate, pH 7.4, 40 mM triethanolamine-HCl neutralized with KOH to pH 7.4, 15 mM potassium succinate, 2 mM potassium glutamate, 2 mM potassium malate, 1 mM K-ATP, 1 mM $MgCl_2$, 1 mM CoA, 1 mM carnitine and 0.65 μg/ml rotenone) described by Stucki and coworkers, (1989), as modified in Berthiaume and coworkers, (1994).

In a 50 μl reaction volume, purified mitochondria (40 μg) and 1X MAB were added to 1.5 ml eppendorf tubes followed by the addition of 5-10 μCi of

[^{125}I]iodopalmitate analog. The labeling was allowed to proceed for 30 minutes at room temperature, then stopped by the addition of 5X SDS PAGE loading buffer and incubation at 95 °C for 2 minutes (Leammli, 1970). 40 μg of protein was separated on a 12 % SDS-PAGE gel according to Leammli (1970). Gels were fixed, stained with Coomassie Brilliant Blue G-250 (Neuhoff *et al*, 1988), destained, dried and radiolabeled proteins were visualized by autoradiography.

In a 50 μl reaction volume, protein fractions were incubated in 20 mM Tris-HCl, pH 7.4, 1 mM DTT buffer and the 1 μCi of [^{125}I]iodopalmitoyl-CoA (Berthiaume *et al.*, 1994) for 30 minutes at 25 °C, then stopped by the addition of 5X SDS PAGE loading buffer and processed as above.

2.8 Fast Performance Liquid Chromatography

2.8.1 Small Scale Purification of p165

2.8.1.1 Preparation of Solubilized Rat Liver Mitochondria

Purified rat liver mitochondria were solubilized in 20 mM Tris-HCl pH 7.4, 0.1 % Nonidet P40 (NP-40), 1 mM PMSF, 1 mM DTT for 30 minutes at 4 °C, and then centrifuged at 12,000 x *g* for 30 minutes at 4 °C in a Beckman JA 20 rotor. The resulting supernatant was adjusted to 20 mM K-MES pH 6.0 (2-[N-Morpholino]ethanesulfonic acid neutralized with KOH) by the addition of a 200 mM K-MES pH 6.0 buffer. The supernatant was dialyzed against S buffer for 2 hours with one exchange of buffer (20 mM K-MES, 1 mM DTT and 1 mM PMSF).

2.8.1.2 SP Sepharose Chromatography

The dialyzed protein solution containing 80 mg of protein was loaded onto a prepackaged 1 ml SP Sepharose HP column pH 6.0, equilibrated with S buffer at a flow rate of 0.5 ml/min. Using a FPLC, a linear NaCl gradient (0-500 mM) was applied to the column over 17 ml and 1 ml fractions were collected. Aliquots of chromatographic fractions were labeled with the [^{125}I]iodopalmitoyl-CoA and analyzed as described above. Fractions containing radiolabeled p165 were pooled.

2.8.1.3 Mono Q Chromatography

The SP Sepharose protein pool was then equilibrated with TD buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT), and applied to a Mono Q HR 5/5 column at a flow rate of 0.5 ml/min. Using a FPLC, the column was washed with 5 column volumes of TD buffer and then eluted stepwise with a 2 ml gradient (0-80 mM NaCl in TD buffer) followed by a 10 ml gradient (80-500 mM NaCl in TD buffer) and one ml fractions were collected. Aliquots of chromatographic fractions were radiolabeled and analyzed by SDS PAGE as described above. Fractions containing radiolabeled p165 were pooled.

2.8.1.4 Hydroxyl Apatite Chromatography

The protein pool from the Mono Q column was loaded onto a 0.5 cm x 5 cm column of Macro-Prep ceramic hydroxyapatite equilibrated in 10 mM K_2HPO_4 pH 6.8. The column was eluted with a 16 ml linear gradient (0-300 mM K_2HPO_4). Aliquots of chromatographic fractions were analyzed by SDS PAGE as described above. Fractions containing radiolabeled p165 were pooled.

2.8.1.5 Phenyl Superose Chromatography

4 M ammonium sulfate solution was added to the protein pool from the hydroxyapatite column to yield a 2 M ammonium sulfate solution and applied to a Phenyl Superose HR 5/5 column equilibrated with 2 M ammonium sulfate in 100 mM K_2HPO_4 buffer (pH 7.0) at a flow rate of 0.5 ml/min using the FPLC. The column was eluted with a linear gradient of 2.0-0 M ammonium sulfate in 100 mM K_2HPO_4 (pH 7.0). One ml fractions were collected. Aliquots of chromatographic fractions were analyzed by SDS PAGE as described above. Fractions containing radiolabeled p165 were frozen at -80 °C with 20 μg/ml leupeptin until use.

2.8.2 Edman Degradation of p165

An apparently homogeneous sample of p165 was sent to the Alberta Peptide Institute (University of Alberta) for Edman degradation analysis. Edman degradation analysis was performed using a HP-G-1005A (Hewlett Packard) protein sequencing system using Version 3.0 Chemistry.

2.8.3 Large Scale Purification of Carbamoyl Phosphate Synthetase 1

Rat liver CPS 1 was isolated according to Powers (1981) with the following changes as the Ultrogel ACA34 gel filtration column was no longer commercially available. Our purification procedure used 3 chromatographic steps instead of one as described by Powers (1981).

2.8.3.1 Preparation of Large Scale Solubilized Rat Liver Mitochondria

Ten male Sprague-Dawley rats (300-350 g each) were anesthetized with sodium pentobarbitol and their livers quickly excised. Rat livers were rinsed with homogenization buffer H (20 mM Tris-HCl pH 7.85, 10 % sucrose, 1 mM PMSF, 1 mM DTT), removed from the buffer and minced into small cubes. A ratio of 3:1 of isolation medium IM (IM: 250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EDTA, 0.1 % albumin (essentially fatty acid free), 1 mM DTT, 1 mM PMSF, 20 μg/ml leupeptin (Powers-Lee and Corina, 1986) to tissue was used and then homogenized using 5 strokes of the glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 x *g* for 5 minutes at 4 °C. The supernatant was filtered through cheese cloth then centrifuged for 30 minutes at 10 400 x *g* at 4 °C in a JA 16 rotor. The pellet was suspended in IM buffer and aliquoted into tubes containing 1 g total mitochondrial protein and stored at -80 °C until use.

For each preparation of CPS 1, a 1 g crude mitochondria aliquot was thawed and swollen in 90 ml hypotonic buffer (1 mM PMSF, 1 mM DTT, 20 μg/μl leupeptin) for 30 min on ice. The suspension was then sonicated twice for 2 min at a setting of 2 using a Heat Systems sonicator. Nine ml of 200 mM Tris pH 7.4 was then added to the solution and the lysed mitochondria were centrifuged at 100 000 x *g* for 60 min at 4 °C in a Beckman Ti 70 rotor. The pellet was discarded.

2.8.3.2 Ammonium Sulfate Precipitation

Solid ammonium sulfate was added to the supernatant to yield a 35 % saturated solution, the extract was stirred for 30 minutes at 4 °C; then centrifuged at 20 500 x *g* for 20 min at 4 °C and the pellet discarded. Solid ammonium sulfate was added to the resulting supernatant while stirring to make it a 80 % saturated solution, the extract was stirred for 30 minutes at 4 °C, and the suspension was centrifuged as above and the resulting supernatant was discarded.

2.8.3.3 Sephacryl-200 Chromatography

The pellet was resuspended in 3 ml of 0.3 M K_2HPO_4 (pH 7.6) and dialyzed for 2 hours against K_2HPO_4 buffer (20 mM K_2HPO_4 pH 7.6, 1 mM DTT, 1 mM PMSF). The protein solution was loaded onto a Hiprep Sephacryl S-200 26/60 equilibrated with 0.3 M K_2HPO_4 (pH 7.6), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 μg/μl leupeptin. The column was eluted with 720 ml of 0.3 M K_2HPO_4 (pH 7.6) at 1 ml/min and 4 ml fractions were collected. Aliquots of chromatographic fractions were subjected to [^{125}I]iodopalmitoyl-CoA labeling, separated by SDS-PAGE as described above and radiolabel incorporation was visualized by phosphorimager analysis. Fractions containing CPS 1, determined by radiolabeled band were pooled.

2.8.3.4 Mono Q Chromatography

The protein pool from the Sephacryl-200 column was equilibrated with TD buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT) then applied to a Mono Q HR 5/5 column. The column was washed with 5 column volumes of TD buffer (pH 8.0)

and then eluted with a 2 ml gradient of 0-80 mM, followed by a 10 ml gradient of 80-300 mM, followed by a 2 ml gradient from 300-500 mM NaCl in TD buffer. Fractions were collected at a flow rate of 0.5 ml/min. Aliquots of chromatographic fractions were subjected to [^{125}I]iodopalmitoyl-CoA labeling, separated by SDS-PAGE as described above and radiolabel incorporation was visualized by phosphorimager analysis. Fractions containing CPS 1, determined by radiolabeled band were pooled.

2.8.3.5 Phenyl Superose Chromatography

The Mono Q protein pool was adjusted to 2 M ammonium sulfate solution and applied to a Phenyl Superose HR 5/5 column equilibrated with 2 M ammonium sulfate in 100 mM K_2HPO_4 buffer (pH 6.5). The column was eluted with a gradient of 2.0-0 M ammonium sulfate in 100 mM K_2HPO_4 pH 6.5. One ml fractions were collected at a flow rate of 0.5 ml/min. Aliquots of chromatographic fractions were subjected to [^{125}I]iodopalmitoyl-CoA labeling, separated by SDS-PAGE as described above and radiolabel incorporation was visualized by phosphorimager analysis. Fractions containing CPS 1, determined by radiolabeled band were frozen at -80 °C with 20 μg/ml leupeptin until use.

2.9 Neutral Hydroxylamine Treatment

Samples (40 μg of mitochondria, 1-2 μg of purified CPS 1, 2.5 μg of bovine glutamate dehydrogenase) were labeled with the [^{125}I]iodopalmitoyl-CoA as described above and loaded in duplicate lanes on a 12 % SDS PAGE on opposite sides of the 2 lanes of prestained molecular weight markers loaded

into lanes in the middle of the gel. Following electrophoresis, the gel was carefully cut in half between the prestained molecular weight marker lanes using a razor blade and each half of the gel was soaked while shaking in either 1 M fresh hydroxylamine (pH 7.0) or 1 M Tris (pH 7.0) for 48 hours. After 48 hours, the gels were rinsed with ddH_20 and stained with Coomasie Brilliant Blue G-250 (Neuhoff *et al*, 1988), destained, and dried. Radiolabel incorporation into proteins was visualized by autoradiography and quantified using ImageQuant 1.2. In the case of rat liver CPS 1, the gels were not fixed before hydroxylamine treatment but this was not the case with bovine liver GluDH and whole rat liver mitochondria.

2.10 Fatty Acid Analysis

2.10.1 Extraction of the lipid label

Fatty acid analysis was performed according to Alland *et al.*, (1994). Purified CPS 1 or GluDH were labeled with the [^{125}I]iodopalmitoyl-CoA as described above and separated on a 12 % SDS PAGE gel. Following electrophoresis, the wet gel was wrapped in Saran Wrap and exposed to x-ray film at -80 °C for 2 days. The gel slice, containing the protein corresponding to the labeled band observed on the autoradiogram, was carefully cut from the gel with a clean razor blade, placed in a glass tube and crushed with a glass rod. 0.5 ml of 1.5 M sodium hydroxide was added and the mixture was allowed to incubate at room temperature for 36 hours. One ml of chloroform and 0.75 ml of 1.0 N HCl were added to the mixture and the tube was vortexed and centrifuged for 5 minutes at 2000 rpm. The chloroform layer was removed and the aqueous

layer was extracted twice with 1.0 ml of chloroform. The combined chloroform extracts were reduced to dryness by an air stream. The residue was analyzed by thin layer chromatography (TLC).

2.10.2 Thin layer chromatography

Lipids were resuspended in 60 µl chloroform and spotted onto a 20 x 20 cm, 250 µM Silcia 60 Gel plates. [^{125}I]iodopalmitate diluted accordingly in chloroform was used as a control. The plates were developed in water/glacial acetic acid/acetonitrile (1:1.75:1.75 v/v/v) (Alland *et al.*, 1994) to separate lipids and dried. Radiolabeled lipids were visualized using phosphorimager analysis while the lipid bands were visualized by iodine vapour.

2.11 NEM Treatment of CPS 1

1-2 µg of purified CPS 1 was preincubated for 30 minutes in the absence (control, 100 mM K-MOPS (3-[N-Morpholino]propanesulfonic acid) pH 7.0) or presence of 1 mM, 2 mM, 5 mM or 10 mM NEM diluted in 100 mM K-MOPS pH 7.0 and then radiolabeled with 0.8 µCi of [^{125}I]iodopalmitoyl-CoA for 30 minutes at 25 °C. Reactions were then stopped by the addition of 5X SDS PAGE loading buffer, proteins were separated on a 12 % SDS PAGE gel and radiolabel incorporation into proteins was visualized by autoradiography.

2.12 CPS 1 Enzymatic Activity

The reaction mixture was prepared as follows: 5 µg of purified CPS 1 was incubated in a reaction mixture containing 20 mM Tris pH 7.4 (Total volume=500 µl) in a spectrophotometer cuvet. In some reactions the sample was preincubated for 30 minutes in the presence of 2, 20, 200 µM palmitoyl-

CoA or CoA or ddH_20. The mixture was then added adjusted with buffer to yield a total of 1 ml and the following composition: 50 mM glycylglycine pH 7.6, 50 mM $KHCO_3$, 35 mM $(NH_4)_2SO_4$, 15 mM $MgSO_4$, 10 mM acetylglutamate, 1.7 mM ATP, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH, 40 μg pyruvate kinase, 200 μg lactate dehydrogenase and 25 μg adenylate kinase (Raijman and Ellen Jones , 1976). The cuvet was then inverted 4 times to ensure adequate mixing and activity was assayed by the oxidation of NADH on a Shimadzu UV 16OU spectrophotometer at Absorbance=340 nm for 10 minutes using the Kinetic Program (Raijman and Ellen Jones, 1976).

2.13 Fatty Acylation of CPS 1 in the Presence of Substrates and Cofactors

1-2 μg of purified CPS 1 was preincubated with saturating concentration of reactants (10 mM AGA, 50 mM HCO_3^-, 35 mM NH_3, 1.7 mM ATP, 4 mM Mg^{2+}) for 30 minutes at 25 °C prior to addition of 0.2 μCi of [^{125}I]iodopalmitoyl-CoA for another 30 minutes at 25 °C. Reactants were first incubated with CPS 1 individually then in various combinations. Reactions were then stopped by the addition of 5X SDS PAGE loading buffer, proteins were separated on a 12 % SDS PAGE gel and radiolabel incorporation into proteins was visualized by autoradiography. Radiolabel incorporation into protein was quantified using ImageQuant 1.2.

2.14 Protein Assay

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

3.0 RESULTS

3.1 Protein Fatty Acylation Patterns in Mitochondria Isolated from Different Tissues

Initial experiments were performed in order to determine whether mitochondrial protein fatty acylation is similar in mitochondria isolated from different tissue origins. Percoll gradient purified mitochondria isolated from different rat tissues were incubated with an [^{125}I]iodopalmitate analog in a cocktail containing cofactors necessary for mitochondrial fatty acid import. We discovered that there were many proteins that could be labeled in this manner in mitochondria isolated from rat heart, liver, brain, kidney and leg gastrocnemius muscle (Fig. 3.1A,B). Radiolabel incorporation into mitochondrial proteins varied among the tissues examined. For example, the overall fatty acylation levels and the number of fatty acylated proteins was highest in the liver and lowest in brain mitochondria. In addition, there were several labeled proteins that were specific to a given tissue. Similar results were also observed when the source of the mitochondria was mouse tissue (Fig. 3.2) except that the overall fatty acylation levels and number of fatty acylated proteins was highest in the liver but lowest in the heart followed by the brain.

It is well known that transformed cells have altered metabolic profiles and often display increased rates of glucose uptake and glycolysis (Dang and Semenza, 1999) as compared to rat hepatocytes. We therefore wanted to examine whether protein fatty acylation is altered in mitochondria after cellular

transformation or immortalization. This was done by comparing mitochondria isolated from rat hepatocytes (normal) and McArdle RH7777, a transformed rat hepatoma cell line (Fig. 3.3A). Overall, the mitochondrial coomassie stained and radiolabeled protein profiles were quite different between the two cell types. Of interest, a prominent 165 kDa protein present in mitochondria isolated from hepatocytes was apparently absent from the hepatoma cell line mitochondria and could be radiolabeled *in vitro* (Fig 3.3-arrow).

3.2 Localization of Rat Liver Mitochondrial Protein Fatty Acylation

Previous results obtained by Stucki and coworkers (1989) demonstrated that mitochondrial protein fatty acylation occurs in the matrix and inner membrane, although mitochondrial outer membrane and intermembrane mitochondrial space isolation was not performed and therefore was not evaluated. Therefore, in order to confirm the localization of mitochondrial fatty acylation under our experimental conditions, rat liver submitochondrial compartments were prepared by the established procedures of Ohlendieck *et al.*, (1986) and Parsons and Williams (1967), then labeled with [^{125}I]iodopalmitoyl-CoA. A variety of marker enzymes were used to assess the purity of the mitochondrial subfractions. These were citrate synthase (matrix) (Srere, 1969), succinate-cytochrome c reductase (inner membrane) (Taylor *et al.*, 1993) and NADH cytochrome c reductase (outer membrane) (Sottocasa *et al.*, 1967). According to the marker assays, all submitochondrial fractions prepared were of greater than 90 % purity. We found that the mitochondrial matrix proteins had the greatest capacity to incorporate label (Fig 3.4, lane 5).

Acylated proteins were also observed in the inner mitochondrial membrane fraction (Fig. 3.4, lane 4). This result confirmed that of Stucki *et al.*, (1989). Interestingly, little to no protein in the outer mitochondrial membrane or the intermembrane mitochondrial space fractions could incorporate label (Fig. 3.4, lanes 2 and 3), suggesting mitochondrial protein fatty acylation occurs primarily in the matrix and the inner membrane of the mitochondria and not in the outer membrane or intermembrane space.

3.3 Hydroxylamine treatment of Whole Rat Liver Mitochondria

To investigate the chemical nature of the radiolabel bound to labeled mitochondrial proteins, whole rat liver mitochondrial protein separated by SDS-PAGE was subjected to hydrolysis with 1 M hydroxylamine for 48 hours. This treatment cleaves thioester bonds but does not affect ester (oxyester) bonds (McIlhinney, 1993). Approximately 70 % of the iodopalmitate label was removed from some of the labeled proteins (* - Fig. 3.5) as determined by ImageQuant analysis, in comparison to the control in which [^{125}I]iodopalmitate labeled whole rat liver mitochondria were treated with 1 M Tris pH 7.0 (Fig. 3.5B). This result suggests that the [^{125}I]iodopalmitate is bound to the majority of the labeled rat liver mitochondrial proteins via a hydroxylamine sensitive thioester bond. No changes were observed in the coomassie blue protein profile as a result of hydroxylamine treatment ensuring that the decrease in label observed reflects removal of [^{125}I]iodopalmitate rather than removal of protein from the gel (Fig. 3.5A).

3.4 Purification of p165 from Rat Liver Mitochondria

As a preliminary step towards understanding the role of protein fatty acylation in the regulation of mitochondrial metabolic function, purification and characterization of fatty acylated mitochondrial proteins is required. In our analysis, we found an abundant 165 kDa fatty acylated protein present in rat liver mitochondria (Fig. 3.1). Radiolabel bound to this 165 kDa protein was removed by hydroxylamine treatment (Fig. 3.5), suggesting that the [^{125}I]iodopalmitate label was bound to the protein via a thioester bond. P165 was also solubilized from mitochondria at low concentrations of detergent, hypotonic shock and sonication (data not shown), suggesting it was not membrane bound. Therefore, we decided that p165 was a good candidate for purification and characterization. Using incorporation of [^{125}I]iodopalmitate into p165 from [^{125}I]iodopalmitoyl-CoA as a functional assay, we purified this 165 kDa protein to apparent homogeneity from rat liver mitochondria as described under the materials and methods (Fig. 3.6).

Edman degradation sequence analysis was performed for the N-terminal fifteen amino acids. A search of the Genbank database revealed 100 % identity with the N-terminal sequence of the mature (processed) rat liver carbamoyl phosphate synthetase 1 (CPS 1) (Table 3.1).

Large scale purification of CPS 1 was adapted from the purification procedure of Powers (1981) with some modification, since the Ultrogel ACA34 gel, which allowed a single step purification of CPS 1, was no longer commercially available. Our purification procedure yielded preparations

greater than 99% purities and with specific activities ranging from 0.20 to 0.43 μmoles carbamoyl phosphate produced per min per mg CPS 1 at 25 °C. The specific activities were lower than those reported by others which varied from 1.87 to 5 μmoles/min/mg of CPS 1 protein (Guthohrlein and Knappe, 1968; Raijman and Jones, 1976; Lusty, 1978; Pierson and Brien, 1980; Elliott and Tipton, 1974a; Clarke, 1976). The increased number of chromatographic steps (three vs one) utilized in our purification protocol may account for those differences. Also, CPS 1 is very susceptible to thermal, oxidative, and proteolytic inactivation (Caravara and Grisolia, 1959; Fahien *et al.*, 1964; Alonso and Rubio, 1987; Guadlajara *et al.*, 1987; Marshall and Fahien, 1988).

3.5 Hydroxylamine Treatment, Fatty Acid Analysis and NEM Treatment of Rat Liver CPS 1

Interestingly, like mitochondrial MMSDH, CPS 1 requires only the fatty acyl-CoA and seems to be autocatalytic *in vitro* with apparently pure preparations. Therefore, to confirm the chemical nature of the bond linking the iodopalmitate to CPS 1 (p165), gel slices containing radiolabeled CPS 1 were subjected to treatment with 1 M hydroxylamine in comparison to the control treated with 1 M Tris. [^{125}I]Iodopalmitate is bound to CPS 1 via an hydroxylamine sensitive thioester bond, since 87 +/- 6 % (n=7) of the radiolabel was removed (Fig. 3.7).

To further explore the nature of the radiolabel bound to CPS 1, we hydrolyzed gel slices containing radiolabeled CPS 1 with 1 M NaOH and analyzed the extract by TLC (Fig. 3.8), in order to detect modifications of the

palmitate. The radioactive label present in the hydrolysate co-migrated with the [^{125}I]iodopalmitate used as a standard. This result confirms that unmodified [^{125}I]iodopalmitate was indeed the chemical entity covalently bound to CPS 1.

It was previously demonstrated that pretreatment of MMSDH (Deichaite *et al.*, 1993) and GluDH (Berthiaume *et al.*, 1994) with N-ethyl maleimide (NEM), which alkylates the sulfhydryl groups of cysteine residues, blocked [^{125}I]iodofatty acyl-CoA binding. Therefore, to confirm that fatty acylation of CPS 1 occurs on a cysteine residue, CPS 1 was preincubated with increasing concentrations of NEM (0 mM (control), 1 mM, 2 mM, 5 mM and 10 mM) prior to incubation with the [^{125}I]iodopalmitoyl-CoA analog (Fig. 3.9). We observed a blockage of [^{125}I]iodopalmitoyl-CoA binding as compared to the control with pretreatment of CPS 1 with all concentrations of NEM (Fig. 3.9). Residual labeling was observed in the NEM treatment lanes (Fig. 3.9). This may be due to hydrophobic interactions between the radiolabel and protein. Overall, this result confirms that fatty acylation of CPS 1 occurs on a cysteine residue(s).

3.6 Inhibition of CPS 1 Catalytic Activity by Palmitoyl-CoA

To this point we have established that CPS 1 is autopalmitoylated *in vitro* and this palmitoylation occurs via a hydroxylamine sensitive thioester bond on a cysteine residue. MMSDH, the only other purified and characterized fatty acylated mitochondrial protein, was shown to be inhibited by physiological concentrations of palmitoyl-CoA (Berthiaume *et al*, 1994). Berthiaume *et al.*, (1994) also demonstrated that bovine liver GluDH may be fatty acylated but the role of its fatty acylation is not known. Interestingly, GluDH enzymatic activity is

also inhibited *in vivo* by submicromolar concentrations of palmitoyl-CoA. Therefore, we wished to investigate the effect of palmitoyl-CoA on CPS 1 activity. We tested CPS 1 enzymatic activity in the presence of increasing concentrations of palmitoyl-CoA. We found that concentrations of palmitoyl-CoA well within physiological mitochondrial concentrations (up to 230 μM) (Faergeman and Knudsen, 1997) could inhibit CPS 1 catalytic activity. In more detail, Fig. 3.10 shows that incubation of CPS 1 with 20 μM palmitoyl-CoA for 30 min inhibited 93.8 +/- 4.5 % (n=8) of CPS 1 catalytic activity, while incubation of CPS 1 with 2 μM palmitoyl-CoA resulted in a lower percentage of inhibition (76.8 +/- 9.4 %, (n=8)) of CPS 1. CPS 1 activity was not affected by 0.2 μM palmitoyl-CoA (6.2 +/- 4.5 % (n=8)) (Fig. 3.10). Similarly, no effect on CPS 1 activity was observed with Coenzyme A concentrations of 0.2, 2 and 20 μM (data not shown). Furthermore, the efficiency of the coupled assay was not impeded by the presence of palmitoyl-CoA in the concentration range tested. These results imply that physiological mitochondrial concentrations of palmitoyl-CoA may inhibit CPS 1 catalytic activity and thus, could play a role in the metabolic regulation of CPS 1.

3.7 Fatty Acylation of CPS 1 in the Presence of Substrates and Cofactors

CPS 1 catalyzes the three step conversion of HCO_3^-, NH_3 and ATP into carbamoyl phosphate in the presence of the allosteric activator n-acetylglutamate (AGA) (Fig. 3.11C). To test whether formation of partial reaction complexes between the enzyme and its substrates, cofactors and allosteric

activator (reactants) would interfere with the fatty acylation process of CPS 1, we preincubated CPS 1 with saturating concentrations of reactants (10 mM AGA, 50 mM HCO_3^-, 35 mM NH_3, 1.7 mM ATP, 4 mM Mg^{2+}), followed by incubation with the [^{125}I]iodopalmitoyl-CoA analog (Fig. 3.11). When CPS 1 was incubated with individual reactants then with [^{125}I]iodopalmitoyl-CoA, we found that AGA treatment almost completely abolished (11.5 % +/- 8.7 % (n=5)) of the control) fatty acylation of CPS 1 while ATP and NH_3 significantly reduced the acylation levels to about 66.0 % +/- 18.2 % (n=5) and 62.5 % +/- 19.2 % (n=4) respectively. By contrast, preincubation of CPS 1 with 50 mM HCO_3^- increased the acylation to 222 % of the control. Addition of reactants pairwise or in combinations yielding formation of either the carboxyphosphate intermediate or the carbamoyl phosphate product had various effects (See CPS 1 three step reaction - Fig. 3.11C). Combination of AGA with either ATP or NH_3 abolished fatty acylation of CPS 1, while additions of reactants to give formation of carboxyphosphate or carbamoylphosphate did not reduce the fatty acylation of CPS 1 and even increased it slightly on average. Together, these results indicate that the fatty acylation site in CPS 1 may be located near the ATP binding site of the catalytic sites or at the AGA allosteric activator binding site of CPS 1. In support of this, the mitochondrial MMSDH is fatty acylated on its active site cysteine residue (Cys319) thereby inhibiting its enzymatic activity (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994).

3.8 Hydroxylamine Treatment and Fatty Acid Analysis of Bovine Glutamate Dehydrogenase

It was previously demonstrated by Kawaguchi and Bloch (1976) that the bovine mitochondrial protein, glutamate dehydrogenase, enzymatic activity was inhibited *in vivo* by micromolar concentrations of palmitoyl-CoA. Also, Berthiaume and coworkers (1994) showed that bovine glutamate dehydrogenase could be fatty acylated and its fatty acylation was prevented by preincubation of the enzyme with N-ethylmaleimide (NEM), a cysteine alkylating agent, thereby suggesting that a cysteine residue is involved in its acylation.

To investigate the chemical nature of the bond linking the iodopalmitate to bovine glutamate dehydrogenase, hydrolysis of bovine glutamate dehydrogenase radiolabeled with [^{125}I]iodopalmitate from gel slices was performed with 1 M hydroxylamine pH 7.0 for 48 hours. As mentioned above, this treatment cleaves thioesters and not ester (oxyester) bonds (McIlhinney, 1993). Compared to the control (Fig. 3.12), 92.5 % +/- 5.7 (n=2) of the iodopalmitate was removed from GluDH (Fig. 3.12). This result indicates that the [^{125}I]iodopalmitate is bound to bovine glutamate dehydrogenase via an hydroxylamine sensitive thioester bond.

Modification of the radiolabel bound to bovine glutamate dehydrogenase was evaluated by hydrolysis of gel slices containing radiolabeled bovine glutamate dehydrogenase with 1 M NaOH, followed by extraction of the neutralized hydrolysate and analysis by TLC. As shown in Fig. 3.13, the radioactive label present in the hydrolysate co-migrated with the

[^{125}I]iodopalmitic acid analog used as a standard. This result suggests that unmodified iodopalmitate was indeed the chemical entity covalently bound to bovine glutamate dehydrogenase. Interestingly, a radiolabled band, migrating just below that of the iodopalmitate was observed when the extracted hydrolysate was analyzed by TLC. This band was present each time the experiment was performed and the identity of this band is unknown.

Fig. 3.1 Fatty acylation patterns of rat mitochondria isolated from different tissues.

40 μg of mitochondrial protein was labeled with 10 μCi of [^{125}I]iodopalmitate in 1X MAB buffer for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 12 % gel. Following electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film for 12 hours at - 80 °C with an intensifying screen. (**A**) Coomassie stained 12 % SDS-PAGE of rat mitochondrial proteins from heart (H), liver (L), brain (B), kidney (K) or leg muscle (LM). (**B**) Autoradiogram of 12 % SDS-PAGE of different mitochondria labeled with [^{125}I]iodopalmitate analog. Molecular weight markers are indicated on the left. Shown are a coomassie stain and the corresponding autoradiograph representative of 8 experiments.

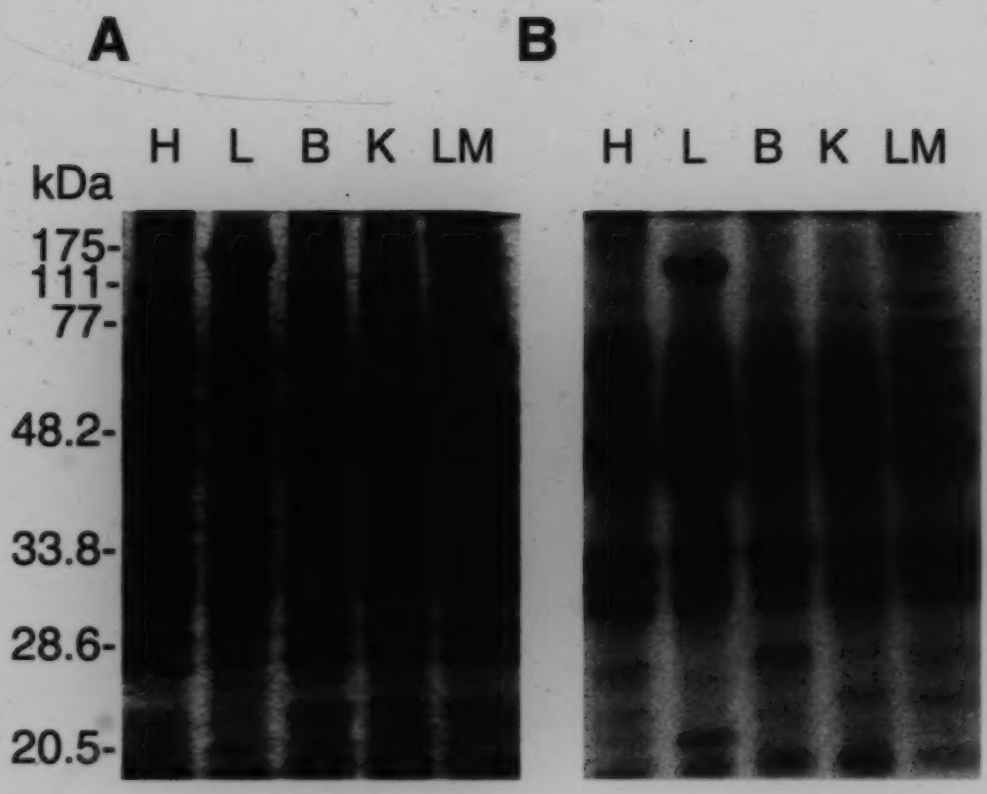

A
B
H L B K LM
H L B K LM
kDa
175-
111-
77-
48.2-
33.8-
28.6-
20.5-

Fig. 3.2 Fatty acylation patterns of mouse mitochondria isolated from different tissues.

40 μg of mitochondrial protein was labeled with 10 μCi of [^{125}I]iodopalmitate in 1X MAB buffer for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved on a 12 % SDS-PAGE gel. Following electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film for 12 hours at - 80 °C with an intensifying screen. (**A**) Coomassie stained 12 % SDS-PAGE of mouse mitochondrial proteins from heart (H), liver (L), brain (B), kidney (K) or leg muscle (LM). (**B**) Autoradiogram of 12 % SDS-PAGE of mitochondria from different tissues labeled with [^{125}I]iodopalmitate analog. Molecular weight markers are indicated on the left. Shown are a coomassie stain and the corresponding autoradiograph representative of 3 individual experiments.

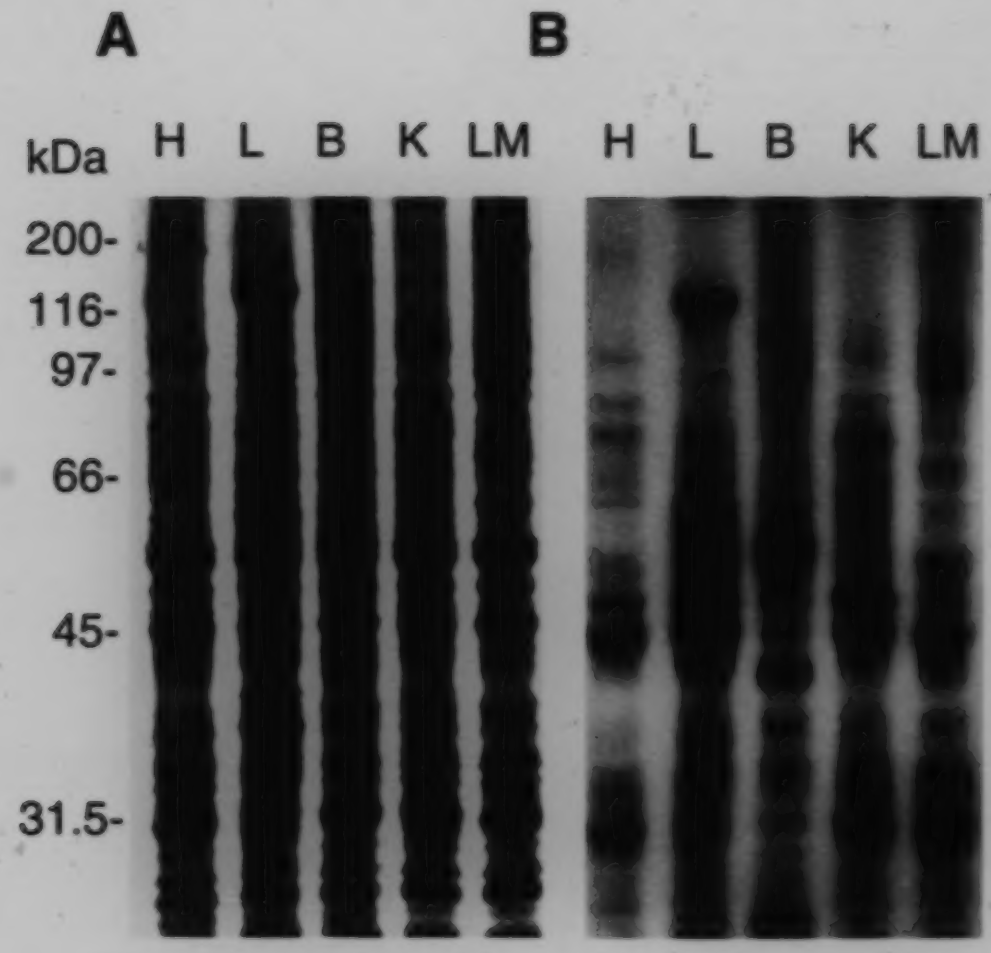

A
B
kDa
H L B K LM
H L B K LM
200-
116-
97-
66-
45-
31.5-

Fig. 3.3 Fatty acylation patterns of mitochondria isolated from transformed and normal hepatocytes.

40 μg of mitochondrial protein was labeled with 10 μCi of [^{125}I]iodopalmitate in 1X MAB buffer for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved on a 8 % SDS-PAGE gel. Following electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film for 12 hours at - 80 °C with an intensifying screen. (**A**) Coomassie stained 8 % SDS-PAGE of mitochondrial proteins isolated from rat hepatocytes (RH) and McA-RH7777 rat hepatoma cells (McA). (**B**) Corresponding autoradiogram of 8 % SDS-PAGE of different mitochondrial proteins labeled with the [^{125}I]iodopalmitate analog. Molecular weight markers are indicated on the left. Arrow indicates the position of p165. Shown are a coomassie stain and the corresponding autoradiograph that is epresentative of 6 experiments.

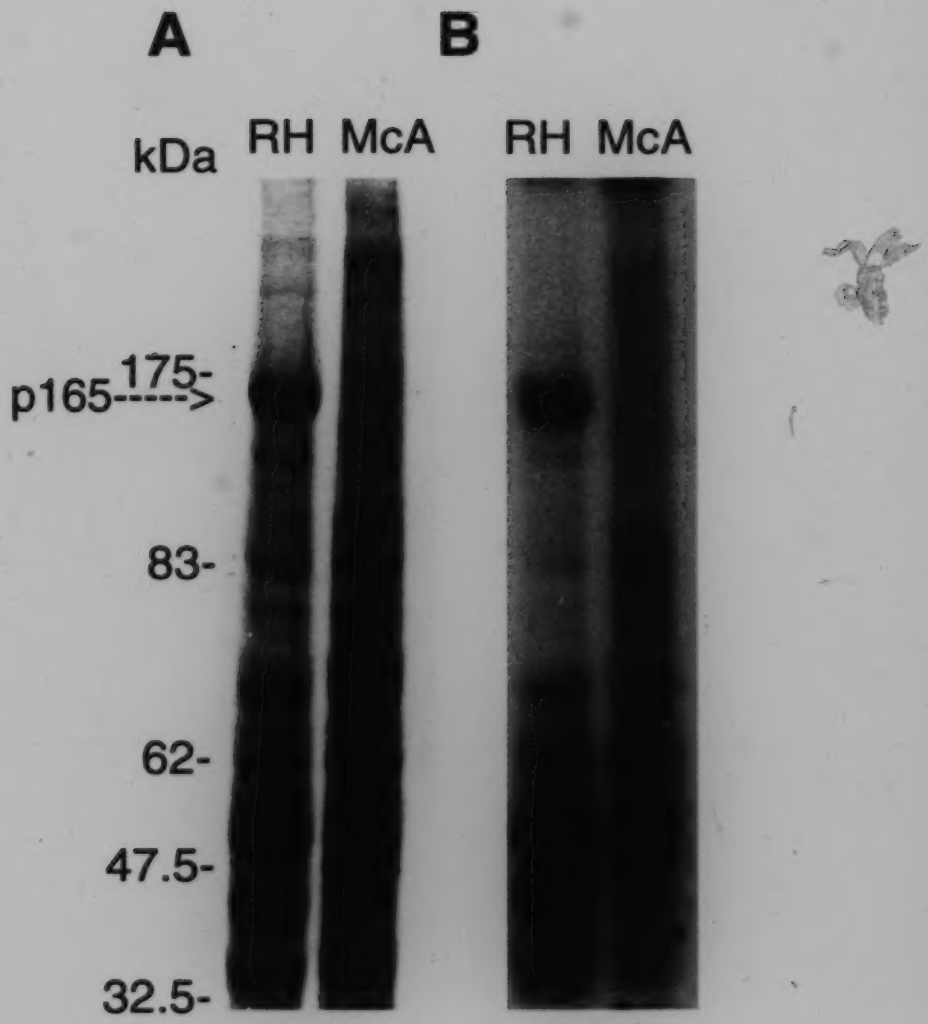

A
B
kDa
RH
McA
RH
McA
p165
175-
83-
62-
47.5-
32.5-

Fig. 3.4 Protein fatty acylation patterns of rat liver submitochondrial compartments.

Preparation of submitochondrial compartments was carried out as described in the Methods. Fractions were radiolabeled with 1 μCi of the [^{125}I]iodopalmitoyl-CoA analog in 20 mM Tris-HCl, pH 7.4 and 1 mM DTT for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 10 % gel. Following electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film. (**A**) Coomassie stained 10 % SDS-PAGE. Lane 1: Rat liver mitochondria, Lane 2: Outer membrane, Lane 3: Intermembrane mitochondrial space, Lane 4: Inner membrane, Lane 5: Matrix. (**B**) Corresponding autoradiogram. Molecular weight markers are indicated on the left. Shown are a coomassie stain and corresponding autoradiograph representative of 4 individual experiments.

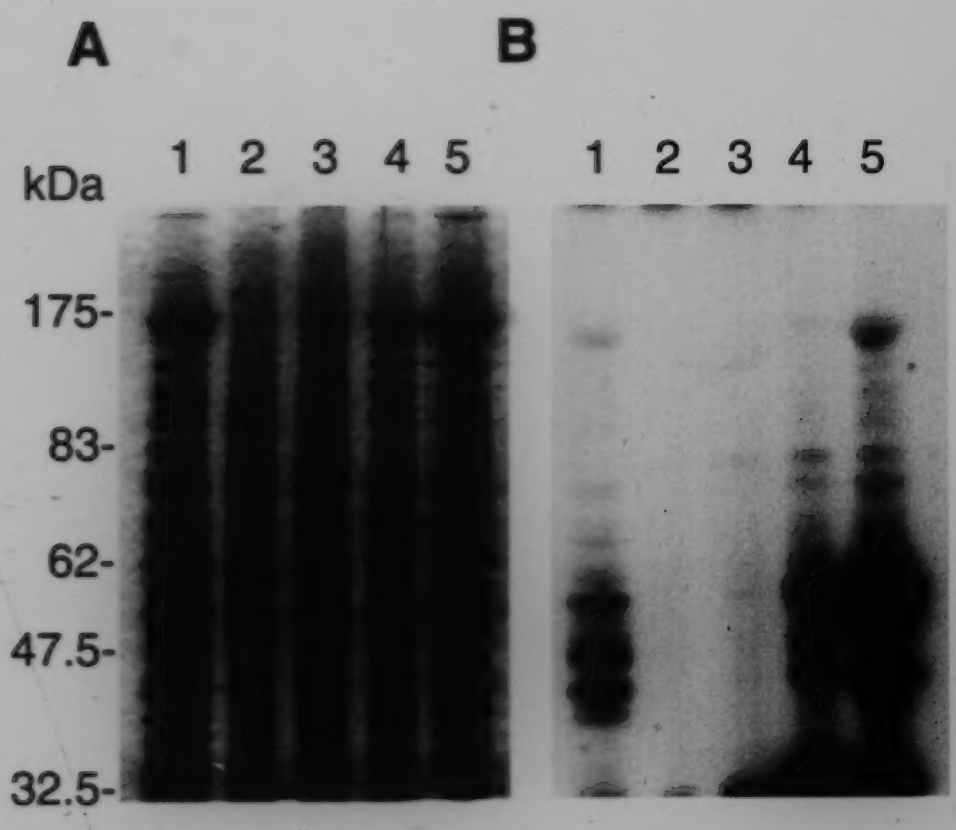

A
B
1 2 3 4 5
1 2 3 4 5
kDa
175-
83-
62-
47.5-
32.5-

Fig. 3.5 Hydroxylamine treatment of [^{125}I]iodopalmitate labeled rat liver mitochondria.

40 μg of purified rat liver mitochondria protein was radiolabeled with 1 μCi of the [^{125}I]iodopalmitate analog in 1X MAB buffer for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 12 % gel. Following electrophoresis, the gel was cut in half and separate lanes were fixed for 2 hours then soaked, while shaking, for 48 hours at 25 °C in either 1 M fresh hydroxylamine pH 7.0 or 1 M Tris pH 7.0. After 48 hours, the gels were rinsed with ddH_20, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film. (**A**) Coomassie stained 12 % SDS-PAGE gel. (**B**) Corresponding autoradiogram. Molecular weight markers are indicated on the left. Arrow indicates the position of p165. Asterisk (*) represents protein labels quantified by ImageQuant 1.2. Shown are the coomassie stain and corresponding autoradiograph representative of 8 experiments.

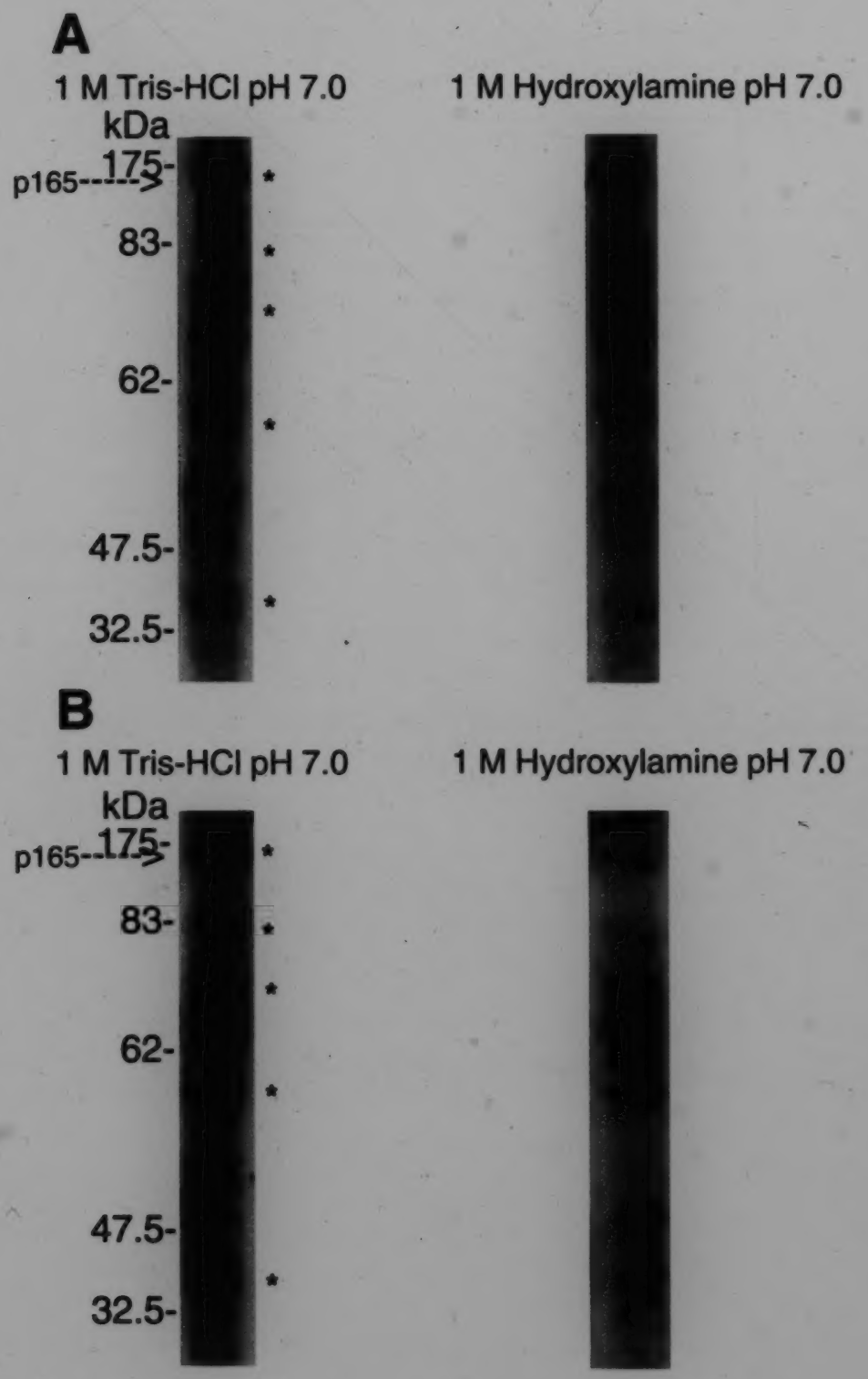

A
1 M Tris-HCl pH 7.0
1 M Hydroxylamine pH 7.0
kDa
175-
p165
83-
62-
47.5-
32.5-
B
1 M Tris-HCl pH 7.0
1 M Hydroxylamine pH 7.0
kDa
175-
p165
83-
62-
47.5-
32.5-

Fig. 3.6 SDS-PAGE analysis of chromatographic fractions containing p165.

Fractions were isolated as described in Methods. Chromatographic fractions were labeled with 1 μCi of the [^{125}I]iodopalmitoyl-CoA analog in 20 mM Tris-HCl, pH 7.4 and 1 mM DTT for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 10 % gel. Following electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue G-250, destained, dried and exposed to phosphorimager analysis. (**A**) Coomassie stained 10 % gel. Lane 1: crude rat liver mitochondria, Lane 2: SP Sepharose pool, Lane 3: Mono Q pool, Lane 4: Hydroxyapatite pool, Lane 5: Phenyl Superose pool. (**B**) Phosphorimager Analysis. Arrow indicates the position of p165. Molecular weight markers are indicated on the left. Shown are the coomassie stain and corresponding autoradiograph representative of 4 individual experiments.

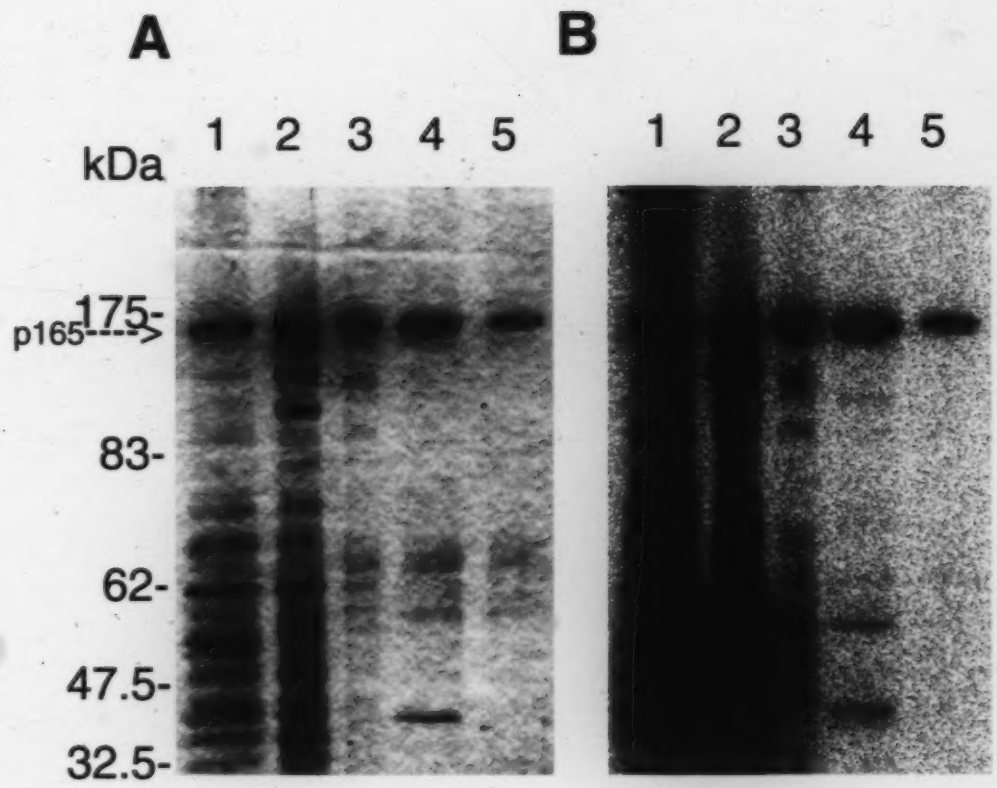

A
B
1 2 3 4 5
1 2 3 4 5
kDa
175-
p165---->
83-
62-
47.5-
32.5-

Table 3.1 Amino acid sequences of the N-terminal fifteen amino acids of p165 and of the mature (processed) rat liver mitochondrial carbamoyl phosphate synthetase 1.

p165 :	S^{1} V K A Q T A H I V L E D G T^{15*}
Rat liver CPS 1:	S^{40} V K A Q T A H I V L E D G T^{54}

* amino acid numbering refers to cycle number in Edman degradation of p165 (1-15) and amino acid number in the processed rat liver mitochondrial CPS 1

Fig. 3.7 Hydroxylamine treatment of [^{125}I]iodopalmitate labeled CPS 1.

1-2 μg of highly purified p165 protein was radiolabeled with 1 μCi of the [^{125}I]iodopalmitoyl-CoA analog in 20 mM Tris-HCl, pH 7.4 and 1 mM DTT for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 12 % gel. Following electrophoresis, the gel was cut in half and separate lanes were then soaked while shaking for 48 hours at 25 °C in either 1 M fresh hydroxylamine (NH_2OH) pH 7.0 or 1 M Tris pH 7.0. After 48 hours, the gels were rinsed with ddH_20 stained with Coomassie Brilliant Blue G-250, destained, dried and exposed to Kodak Biomax MS film. Residual radiolabel was detected by autoradiography. (**A**) Coomassie stained 12 % gel. (**B**) Corresponding autoradiogram. Molecular weight markers are indicated on the left. Shown are a coomassie stain and the corresponding autoradiograph which is representative of 7 individual experiments.

A

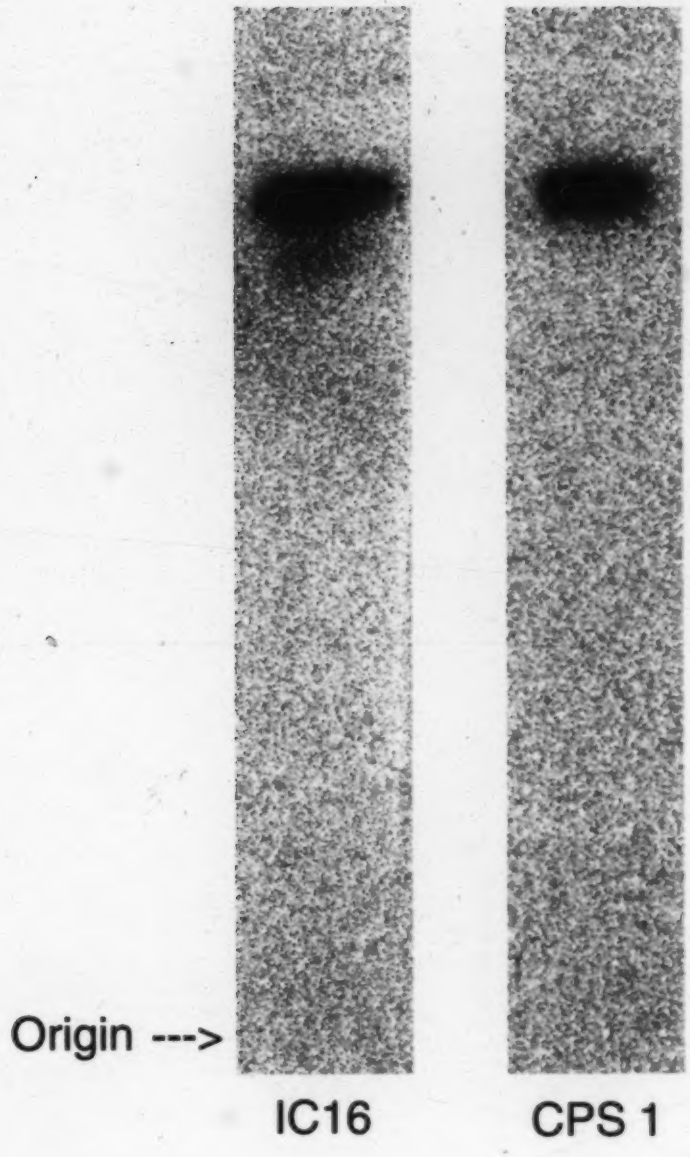
1 M Tris-HCl pH 7.0
1 M Hydroxylamine pH 7.0
kDa
175-

B

1 M Tris-HCl pH 7.0
1 M Hydroxylamine pH 7.0
kDa
175-

Fig. 3.8 Thin layer chromatography analysis of fatty acid hydrolyzed from CPS 1.

Gel slices containing the radiolabeled p165 were hydrolyzed as in the Methods and extracted with chloroform. The chloroform extract was reduced to dryness by an air stream and the residue was analyzed by TLC on 250 μM Silica Gel 60 plates developed in water/glacial acetic acid/acetonitrile (1:1.75:1.75 v/v/v) beside an [^{125}I]iodopalmitate standard. Following air drying, phosphorimager analysis was performed to detect the radiolabeled lipid. Shown is the TLC representative of 4 experiments. ICI6 = [^{125}I]iodopalmitate



IC16 CPS 1

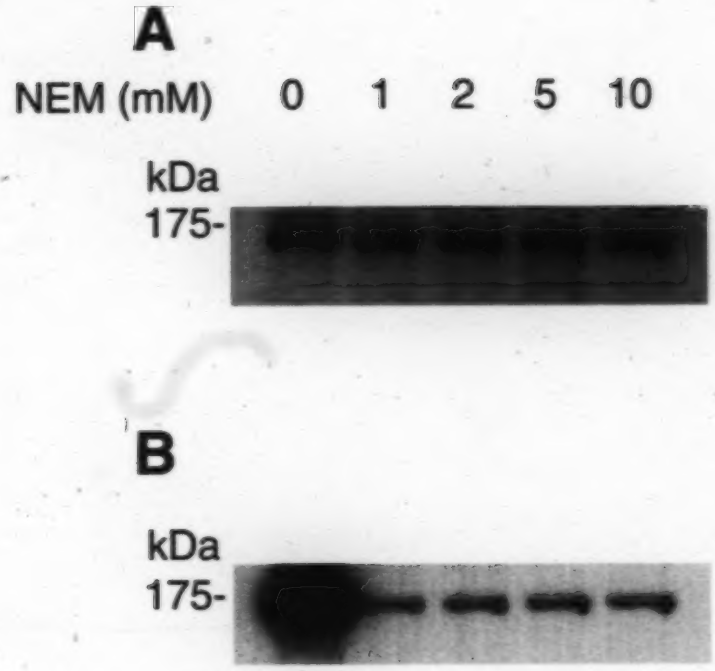


Fig. 3.9 NEM treatment of CPS 1.

1-2 μg of highly purified CPS 1 was preincubated for 30 minutes in the absence (control) or presence of 1 mM, 2 mM, 5 mM or 10 mM NEM and then radiolabeled for 30 minutes as described in the Methods. Proteins were separated on a 12 % SDS-PAGE gel and visualized by autoradiography as described in the Methods. (**A**) Coomassie stained SDS-PAGE. (**B**) Autoradiogram of the 12 % SDS-PAGE of CPS 1 labeled with the [^{125}I]iodopalmitoyl-CoA analog. Molecular weight markers are indicated on the left. Shown are the coomassie stain and corresponding autoradiograph representative of 4 experiments.

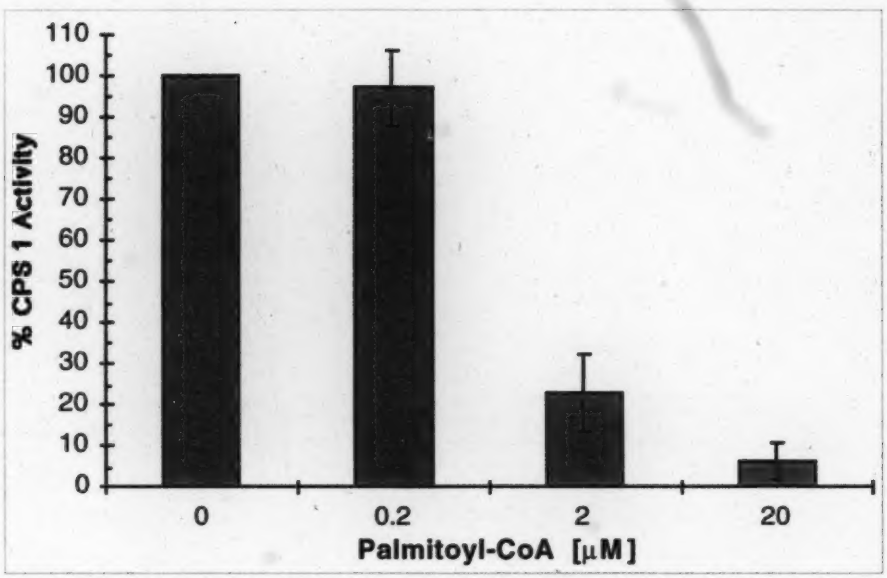
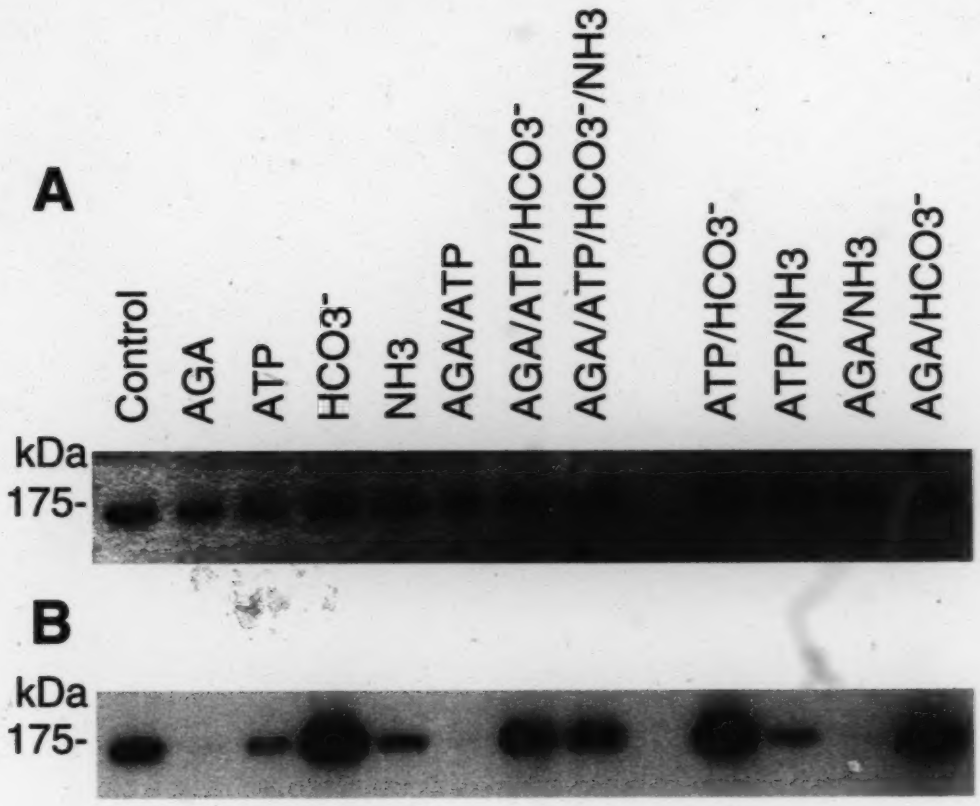


Fig. 3.10 Characterization of CPS 1 enzymatic activity in the presence of palmitoyl-CoA.

Residual CPS 1 enzymatic activity which was preincubated in the presence of 0.2, 2 and 20 μM palmitoyl-CoA for 30 minutes and then assayed as described in the Methods. Values are presented as percentage of control (no palmitoyl-CoA, 100%) values. Each value represents the mean ± SD of 8 experiments.

Fig. 3.11 Characterization of CPS 1 fatty acylation in the presence of various CPS 1 substrates and substrate combinations.

CPS 1 was preincubated for 30 minutes in the presence of various CPS 1 substrates (10 mM AGA, 50 mM HCO_3^-, 35 mM NH_3, 1.7 mM ATP) or substrate combinations and then labeled for 30 minutes as described in the Methods. Proteins were separated on a 12 % SDS-PAGE gel and visualized by autoradiography as described in the Methods. (**A**) Coomassie stained SDS-PAGE. (**B**) Autoradiogram of the 12 % SDS-PAGE of CPS 1 labeled with the [^{125}I]iodopalmitoyl-CoA analog. Molecular weight markers are indicated on the left. Shown are the coomassie stain and corresponding autoradiograph representative of 5 individual experiments. (**C**) CPS 1 reaction mechanism.



C

Step 1: $\xrightarrow{\text{AGA}}$ MgATP + HCO_3^- ---> MgADP + carboxyphosphate

Step 2: carboxyphosphate + NH_3 ---> Pi + carbamate

Step 3: carbamate + MgATP ---> MgADP + carbamoylphophate

$\xrightarrow{\text{CPS 1, AGA}}$ 2MgATP + NH_3 + HCO_3^- ------> 2MgADP + Pi + carbamoylphosphate

Fig. 3.12 Hydroxylamine treatment of [^{125}I]iodopalmitate labeled bovine glutamate dehydrogenase.

2.5 μg of commercial bovine glutamate dehydrogenase protein was radiolabeled with 1 μCi of the [^{125}I]iodopalmitoyl-CoA analog in 20 mM Tris-HCl, pH 7.4 and 1 mM DTT for 30 minutes at 25 °C. Reactions were terminated by the addition of 5X SDS-PAGE loading buffer and incubation at 95 °C for 2 minutes. Samples were resolved by SDS-PAGE on a 12 % gel. Following electrophoresis, the gel was cut in half and separate lanes then soaked, while shaking, for 48 hours at 25 °C in either 1 M fresh hydroxylamine (NH_2OH) pH 7.0 or 1 M Tris pH 7.0. After 48 hours, the gels were rinsed with ddH_20, stained with Coomassie Brilliant Blue G-250, destained, and dried. Radiolabeled proteins were visualized by autoradiography using Kodak Biomax MS film. (**A**) Coomassie stained 12 % gel. (**B**) Corresponding autoradiogram. Molecular weight markers are indicated on the left. Shown are a coomassie stain and the corresponding autoradiograph that is representative of 2 individual experiments.

A

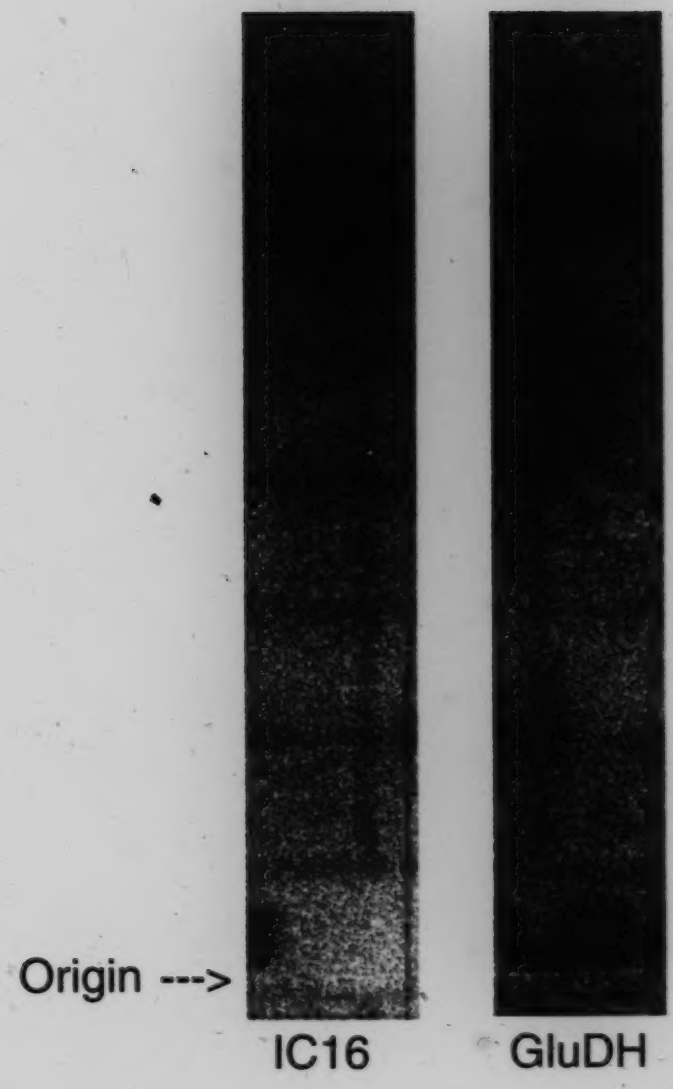
1 M Tris-HCl pH 7.0 1 M Hydroxylamine pH 7.0

B

1 M Tris-HCl pH 7.0 1 M Hydroxylamine pH 7.0

Fig. 3.13 Thin layer chromatography analysis of fatty acid hydrolyzed from bovine glutamate dehydrogenase.

Gel slice containing the radiolabeled bovine glutamate dehydrogenase was hydrolyzed as described in the Methods and extracted with chloroform. The chloroform extract was reduced to dryness and the residue was analyzed by TLC on 250 μM Silica Gel 60 plates developed in water/glacial acetic acid/acetonitrile (1:1.75:1.75 v/v/v) beside an [^{125}I]iodopalmitate standard. ICI6 = [^{125}I]iodopalmitate. Following air drying, phosphorimager analysis was performed in order to visualize radiolabeled lipid. Shown is the TLC representative of 5 experiments.


Origin --->
IC16
GluDH

4.0 DISCUSSION

4.1 Protein Fatty Acylation Patterns in Mitochondria Isolated from Different Tissue Origins

Our laboratory employed the iodoanalogs of palmitate and palmitoyl-CoA, [^{125}I]iodopalmitate and [^{125}I]iodopalmitoyl-CoA, to aid in the identification of fatty acylated (palmitoylated) proteins within the mitochondria. To our knowledge there has been only three other reports by investigators researching this area; one report in 1989 by Stucki and coworkers, in which fatty acylation in rat liver mitochondria was investigated and the two other reports centered around the purification and identification of the fatty acylated liver mitochondrial protein MMSDH (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). Initially, we decided to characterize the general occurrence of mitochondrial protein fatty acylation. Therefore, we analyzed protein fatty acylation in mitochondria isolated from different tissues. We found that a unique set of proteins were fatty acylated in mitochondria isolated from each of the different rat tissues; heart, liver, brain, kidney and leg gastrocnemius muscle. The overall fatty acylation levels and number of fatty acylated proteins was highest in the liver and lowest in the brain, with each tissue having several specific labeled proteins. In general, similar fatty acylation patterns were observed when the source of the mitochondria was mouse tissue. In the mouse tissues, the overall fatty acylation levels and number of fatty acylated proteins was highest in the liver. In contrast to the rat, lowest levels occurred in the heart followed by the brain. The reason for this difference in fatty acylation is not known. These different protein fatty

acylation patterns may reflect tissue specific expression of proteins that vary in their susceptibility to fatty acylation, either enzymatically or autocatalytically. For example, the 165 kDa fatty acylated protein purified from rat liver mitochondria was identified here as carbamoyl phosphate synthetase 1, a protein involved in the urea cycle (Ratner, 1973; Krebs *et al.*, 1973, Tatibana and Shigesada, 1976). It is present only in liver and small intestine mitochondria (Gamble and Lehninger, 1973; Clarke, 1976). Secondly, the different protein fatty acylation patterns may reflect differences in the metabolic requirement of the individual mitochondria.

Protein fatty acylation patterns were also examined in mitochondria after cellular transformation or immortalization through the comparison of mitochondria isolated from rat hepatocytes (normal) and McArdle RH7777 cells, McArdle RH7777 are a transformed rat hepatoma cell line. A unique set of proteins were fatty acylated in mitochondria isolated from transformed cells when compared to those isolated from rat hepatocytes. Again, the different protein fatty acylation patterns may reflect the expression of different proteins that vary in their susceptibility to fatty acylation due to differences in metabolism in these mitochondria. For example, transformed cells generally have altered metabolic profiles and often display increased rates of glucose uptake and glycolysis (Dang and Semenza, 1999) as compared to rat hepatocytes.

Further analysis of fatty acylated protein content in rat mitochondrial subcompartments indicated that the majority of proteins that could be acylated reside in the matrix with fewer localized to the inner membrane. This supports

the claim made by Stucki *et al.*, (1989) who also showed that the majority of acylated mitochondrial proteins were in the matrix and inner membrane fractions. However, the mitochondrial outer membrane and intermembrane space mitochondrial isolation was not performed. We show here that very few if not any proteins are acylated in these compartments. Indeed, the mitochondrial matrix and inner membrane are important sites for the catabolism of various substrates needed for energy production (Moran *et al.*, 1994). By extension, it is conceivable that fatty acylation of proteins in these compartments may reflect a novel type of metabolic regulation mechanism.

4.2 Identification and characterization of fatty acylation of new mitochondrial proteins

It has been demonstrated previously, in rat liver and COS-7 cells, that several mitochondrial proteins can incorporate fatty acids in a covalent manner (Stucki *et al.*, 1989; Berthiaume *et al.*, 1994). However, to date only one of these fatty acylated mitochondrial protein has been identified, MMSDH (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). Berthiaume and coworkers, (1994) proposed a model in which MMSDH's fatty acylation could act as a novel mode of regulation of its enzymatic activity within the mitochondria, suggesting the possibility of a metabolic cross-talk between amino acid and fatty acid catabolic pathways (Berthiaume *et al.*, 1994). In order to determine if protein fatty acylation is a general mechanism of metabolic regulation in the mitochondria, we needed to characterize the role of protein fatty acylation in other mitochondrial proteins.

4.2.1 Carbamoyl phosphate synthetase 1

We observed that a major radiolabeled protein of 165 kDa was present in mitochondria isolated from rat liver, mouse liver and rat liver hepatocytes but absent from mitochondria isolated from transformed rat hepatocytes. Therefore we purified this protein using chromatographic techniques. Amino acid sequence analysis and an inquiry to the Genbank database revealed that p165 was rat liver mitochondrial carbamoyl phosphate synthetase 1 (CPS 1). CPS 1 is involved in the distal part of amino acid catabolism, where it catalyses the first and rate limiting step of urea synthesis (Ratner, 1973; Krebs *et al.*, 1973; Tatibana and Shigesada, 1976). CPS 1 has been reported to be absent from a number of hepatoma cell lines, however, it has also shown to be present in others (Lawson *et al.*, 1975; Irwin *et al.*, 1978). We now further these observations by showing that p165 is absent in the transformed rat hepatoma cell line, McA RH7777. The significance of this observation and that of others, still remains to be investigated.

Confirmation that CPS 1 is covalently modified on a cysteine residue(s) with iodopalmitate via a thioester bond was obtained by its sensitivity to cleavage by neutral hydroxylamine, chromatographic characterization of the liberated iodopalmitate and blockage of [^{125}I]iodopalmitoyl-CoA binding by pretreatment of CPS 1 with the alkylating agent NEM. CPS 1 is now the second mitochondrial protein that has been shown to be fatty acylated. Berthiaume *et al.*, (1994) reported earlier that MMSDH is covalently fatty acylated at its active site cysteine residue and perhaps more importantly, showed that this

modification abolishes MMSDH activity. In a similar manner, we have shown here that the fatty acylation of CPS 1 apparently results in a loss of enzymatic activity.

In order to gather more information on the possible palmitate acceptor site(s) of CPS 1, we incubated CPS 1 with various substrates, cofactors and its allosteric activator. Since the AGA allosteric activator, the ATP cofactor and the NH_3 substrate of CPS 1 either apparently abolished fatty acylation or significantly reduced it, we believe that acylation either occurs at or near the allosteric AGA binding site or at one (or both) of the active sites of CPS 1. In support of the active site being the site of acylation, there is a 17 amino acid sequence surrounding Cys1327 of CPS 1 that has similarity to the surrounding acylated active site cysteine of MMSDH (Cys 319) (Table 4.1). According to studies performed by Potter and Powers-Lee (1992), Cys1327 of CPS 1 was reported to be part of the ATP site resulting in the formation of carbamate and it is close to the part of AGA site present in domain D of the protein. Also, it was demonstrated that cysteines 1327 and 1337 showed increased reactivity of thiol groups in the presence of AGA (Geshwill and Lumper, 1989). Therefore, fatty acylation of Cys1327 could potentially account for the inhibition of CPS 1 by concentrations of palmitoyl-CoA that are within physiological range (below 230 μM) (Faergeman and Knudsen, 1997). Alternatively, a conformational change could occur in CPS 1 in the presence of AGA as suggested by Rubio *et al.*, (1983) and therefore allowing access to certain substrates which could prevent the fatty acylation of the acyl-acceptor cysteine residue(s). Interestingly,

preincubation of CPS 1 with either HCO_3^- alone or various substrate combinations, leads to an increase in fatty acylation levels. While HCO_3^- may act as a weak base and stimulate acylation of CPS 1, the increases observed in the presence of various combinations of reactants remains to be examined further.

4.2.2 GluDH

Kawaguchi and Bloch (1976) first demonstrated that bovine liver GluDH enzymatic activity was inhibited by micromolar concentrations of palmitoyl-CoA. In 1994, Berthiaume and coworkers demonstrated that bovine liver GluDH could bind fatty acid covalently and that fatty acylation could be prevented by preincubation of the enzyme with NEM, an alkylating agent, thereby suggesting that a cysteine residue was involved in its acylation. The nature of the linkage of the bound fatty acid was not identified. We now show that the protein is covalently modified on a cysteine residue(s) with iodopalmitate, since iodopalmitate was liberated after treatment with neutral hydroxylamine. This data also indicates that the iodopalmitate protein linkage occurs via a thioester bond. GluDH is now the third mitochondrial protein that has been confirmed to be fatty acylated.

4.3 Autoacylation

The fatty acylation of mitochondrial bovine liver MMSDH apparently required only the fatty acyl-CoA moiety and seemed to be autocatalytic *in vitro* in apparently pure preparations of MMSDH (Deichaite *et al.*, 1993). Autoacylation was also observed with bovine liver GluDH (Berthiaume *et al.*,

1994) and CPS 1 (work herein). In support of this concept, overexpressed recombinant bovine liver MMSDH prepared from *E. coli* lysates were readily fatty acylated in the presence of [I^{125}]iodopalmitoyl-CoA (L.G.B. unpublished data). These data suggest that a mitochondrial "transferase" may not be necessary for fatty acylation of bovine liver mitochondrial MMSDH. Preparations of mitochondrial rat liver CPS 1 used in our fatty acylation assays were greater than 99 % homogeneous and unless a minute amount of a given transferase is present in our CPS 1 preparations, fatty acylation of CPS 1 may also be autocatalytic. To date, many proteins have been shown to be fatty acylated non-enzymatically such as: G protein α subunits (Duncan and Gilman, 1996), mylein proteolipid protein (PLP) (Bizzozero *et al.*, 1987; Bizzozero *et al.*, 1990), mylein P_o glycoprotein (Bharadwaj and Bizzozero, 1995), rhodopsin (O'Brien *et al.*, 1987), Semliki Forest virus E2 glycoprotein (Berger and Schmidt, 1984), UDP-glucuronosyl transferase isoforms (UDGPT) (Yasmashita *et al.*, 1995), porcine pancreatic phospholipase A_2 (Tomasselli *et al.*, 1989) and methylmalonyl semialdehyde dehydrogenase (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). The physiological relevance of this process remains unclear. In the cases of the myelin proteolipid protein, myelin P_o glycoprotein and the Giα1 protein, autocatalytic fatty acylation occurred at the same sites as those that became palmitoylated enzymatically *in vivo* (Bizzozero *et al.*, 1987; Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). This observation raises the question for the requirement of an enzyme. It is not known if there is PAT activity present in the mitochondria.

4.4 A role for fatty acylation of mitochondrial proteins

4.4.1 Metabolic Cross-Talk

Previous functions of protein palmitoylation have included subcellular targeting (or localization) of proteins to membranes and organelles (Wolven *et al.*, 1997; Gonzola and Linder, 1998; Bijlmakers *et al.*, 1997; Melkonian *et al.*, 1999), sorting of proteins (Breuer and Brulke, 1998), mediation of protein-protein interactions involving activation (Ponimaskin *et al.*, 1998) or inhibition (Nakamura *et al.*, 1998; Sudo *et al.*, 1992), regulation of membrane receptor endocytosis and downregulation (Bouvier, 1995), regulation of enzymatic activity (Berthiaume *et al.*, 1994), and virus budding (Ivanova and Schlesinger, 1993). But, an interesting concept emerging from this research is that protein fatty acylation may function in metabolic cross-talk between the fatty acid catabolic pathway and either amino acid oxidation or the urea cycle. Berthiaume and coworkers (1994) proposed a model suggesting that the fatty acylation of MMSDH could act as novel mode of enzymatic activity regulation within the mitochondria and therefore suggests the possibility of a metabolic-cross talk between amino acid and fatty acid catabolic pathways. Berthiaume and coworkers (1994) also demonstrated that mitochondrial bovine liver GluDH was fatty acylated. In addition, this work demonstrates that rat liver CPS 1 is fatty acylated and further confirms that bovine liver GluDH is fatty acylated. There is evidence to support the possibility of a metabolic cross-talk within the mitochondria: 1. ammonia has been shown to inhibit fatty acid oxidation while no effect was observed with the oxidation of succinate or malate and glutamate

(Maddaiah, 1985; Maddaiah and Miller, 1989), 2. patients suffering from Reye's syndrome often exhibit hyperammonemia and display microvesicular fatty metamorphosis of the liver (Saudubray and Charpenter, 1995), 3. patients or animals with genetic alteration of enzymes involved in the β-oxidation cascade (e.g. MCAD or LCAD) often suffer from hyperammonemia (Roe *et al.*, 1995) and 4. excess fatty acid oxidation has been documented to decrease the oxidation of deaminated branched chain amino acids (Leu, Ile, Val) (Aoki *et al.*, 1975b; Cahill, 1976b). Our model describing the role of mitochondrial protein fatty acylation in coordination of substrate degradation in the liver mitochondria in the fed or fasted state is shown in Fig. 4.1A and 4.1B. These observations along with our data suggests the following model for substrate degradation in the liver mitochondria in which protein fatty acylation would play a role.

In the fed state, metabolic energy is derived mostly from the tricarboxylic cycle (Krebs cycle) and typically excess amino acids are utilized to derive metabolic energy or for glucose or fatty acid synthesis. Excess glucose and fatty acids are stored as glycogen or triacylglycerol (TG) respectively. Also, palmitoyl-CoA the acyl donor for both enzymatic and nonenzymatic (autoacylation) palmitoylation of proteins, inhibits several mitochondrial enzymes including: rat adipocyte pyruvate dehydrogenase (PDH) (Moore *et al.*, 1992), rat liver ADP/ATP translocase (AAT) (Morel *et al.*, 1974), bovine liver glutamate dehydrogenase (GluDH) (Kawaguchi and Bloch, 1976), and bovine liver methylmalonyl semialdehyde dehydrogenase (MMSDH) (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). The metabolic interrelations between

predominant catabolic pathways in the mitochondria in the fed state are summarized in Fig. 4.1A. In the transition from the fed state to the fasted state, the body adapts through a series of metabolic alterations which include glycoenolysis (1-2 days), gluconeogenesis (first week), ketosis (3-4 days onward) and finally a progressive decrease in gluconeogenesis which is accompanied by an increased cerebral ketone bodies consumption (Cahill, 1976b).

In the fasted state, glycoenolysis, gluconeogenesis and ketogensis predominate in the liver. Progressively, utilization of amino acids for gluconeogenesis is reduced while the body relies more and more on fatty acid oxidation to derive metabolic energy. While the liver is flooded with fatty acids, reducing equivalents resulting from their oxidation and acetyl-CoA could inhibit β-oxidation. Excess acetyl-CoA moieties are exported from the liver as ketone bodies. This reduction in amino acid utilization is essential for survival as it spares vitally needed proteins. This nitrogen sparing strategy is accompanied by a 60 to 80% diminution in urinary nitrogen secretion (Cahill, 1976a; Owen *et al.*, 1969; Owen *et al.*, 1967). Interestingly, most of this reduction comes from a 75 to 95 % reduction in urea synthesis (Cahill, 1976a; Owen *et al.*, 1969; Owen *et al.*, 1967). In that case, palmitoyl-CoA dependent inhibition of CPS 1, which catalyzes the rate limiting step of urea synthesis (Ratner, 1973; Krebs *et al.*, 1973; Tatibana and Shigesada, 1976), could account for part of the reduction in urea cycle activity. Thus, as suggested in our model, the "switch" responsible for a reduction in amino acid oxidation and urea synthesis activity could be

mediated by fatty acylation and inhibition of a few key metabolic enzymes (Fig. 4.1B). Fatty acylation of these enzymes could contribute to the nitrogen sparing effect observed during starvation.

In our model, in the fasted state (Fig. 4.1B), concerted inhibition of the mitochondrial enzymes; MMSDH, CPS 1, GluDH and pyruvate dehydrogenase (PDH) by palmitoyl-CoA would lead to an accumulation of glutamate and ammonia in the mitochondria and a stimulation of gluconeogenesis. In addition, accumulation of acetyl-CoA from β-oxidation of fatty acids would lead to feedback inhibition of β-oxidation cascade, increased mitochondrial concentration of fatty acyl-CoAs, and inhibition of PDH and stimulation of ketone body production. Excess glutamate or ammonia predicted to be produced by our model (*e.g.* in periportal hepatocytes) would then be transformed locally into glutamine by perivenous hepatocytes, which are rich in glutamine synthetase, or by skeletal muscle cells (Haussinger, 1990; Moran *et al.*, 1994; Jungas *et al.*, 1992; Meijer *et al.*, 1990). These predictions are physiologically consistent with the established fact that the liver has a net production of glutamate (Cahill *et al.*, 1981) and glutamine (Ishikawa, 1976) during starvation.

CPS 1 fatty acylation levels in the presence of either AGA, NH_3 or ATP were significantly reduced and this can be interpreted as a safety mechanism to prevent noxious accumulation of toxic ammonia. Indeed, when amino acids and fatty acids are utilized as a source of energy early in starvation, large amounts of acetyl-CoA, glutamate, ammonia and ATP start to accumulate. As the levels of AGA are controlled by the AGA synthase, which utilizes acetyl-CoA

and glutamate as substrates, an increase in the levels of the latter two substrates will lead to an increase in production of AGA (Moran *et al.*, 1994). This increase in concentration of allosteric activator AGA will activate CPS 1, prevent fatty acylation of CPS 1 preventing any inhibition. In addition, during amino acid catabolism, concentration of liver mitochondrial ammonia increases via the action of either local asparaginase, glutaminase or glutamate dehydrogenase. Higher liver mitochondrial ammonia levels could also contribute to reduce the fatty acylation levels of CPS 1, thus increasing its activity and increasing the supply of carbamoylphosphate for the urea cycle. Higher levels of ATP, an indicator of higher mitochondrial energy charge, may also contribute to the negative regulation of the fatty acylation rate of CPS 1 and therefore stimulate production of carbamoylphosphate. As such, the negative regulation of CPS 1 fatty acylation by AGA, NH_3 and ATP could act as a safety mechanism (Fig. 4.1C).

In the absence of inhibition of fatty acylation of CPS 1 by AGA, NH_3 and ATP, the inhibition of CPS 1 by 20 μM palmitoyl-CoA *in vitro* may appear to be contradictory as our results predict that the enzyme should be completely inhibited under physiological conditions. Indeed, the concentrations of long chain fatty acyl-CoA in liver mitochondrial during the fed state has been estimated at 230 μM (Faergeman and Knudsen, 1997) and is postulated to increase as much as threefold in response to increases in serum free fatty acid concentration from 0.66 mM to 1.60 mM during starvation (Owen *et al.*, 1969). Nonetheless, CPS 1 is an abundant mitochondrial protein, it represents 15-26

% of mitochondrial matrix protein and its concentration is estimated at 0.4 to 1.5 mM (Meijer *et al.*, 1990). As such, concentration of long chain acyl-CoAs in the mitochondria in the fasted state would be sufficient to inhibit a large proportion of CPS 1 but potentially not all of it. This observation could thus explain the partial but not total reduction in urea secretion observed during starvation (Cahill, 1976; Owen *et al.*, 1969; Owen *et al.*, 1967) .

In our model depicting palmitoylation dependent metabolic cross-talk between amino acid and fatty acid catabolic pathways (Fig. 4.1), palmitoylation of MMSDH and CPS 1 (and likely GluDH) could down regulate the degradation of up to 18 amino acids and three nucleotides (Salway, 1994), thus somewhat explaining the reduction in urea synthesis in the fasted state. Furthermore, accumulation of long chain acyl-CoAs could play a role in some pathophysiological states such as in those found in patients suffering from VLCAD or LCAD deficiencies and even in the ischemic heart injury where mitochondrial concentrations of palmitoyl-CoA have been reported to be as high as 1.0 mM (Idell-Wenger *et al.*, 1978). Such high concentrations of palmitoyl-CoA in the heart could thus inhibit several catabolic pathways and hinder energy production.

4.4.2 Potential Targeting to Membranes for Degradation

Another role for fatty acylation of the rat liver mitochondrial enzyme CPS 1 is that it could target the enzyme to the mitochondrial inner membrane for degradation. The only protein known in which palmitoylation may target the protein for degradation is in the case of SCG10, a soluble cytosolic neuronal

growth-associated protein (Di Paolo *et al.*, 1997). The dually palmitoylated protein is more susceptible to proteolytic cleavage in the Golgi apparatus than the mutant forms of the enzyme (Di Paolo *et al.*, 1997). The urea cycle in the liver involves five enzymes, two of which are found in the mitochondria and the rest reside the cytosol. The urea cycle mitochondrial enzymes CPS 1 and ornithine transcarbamoylase have been shown to be loosely associated with the inner mitochondrial membrane (Powers-Lee *et al.*, 1987) even though previous work has demonstrated that both enzymes are contained completely within the inner mitochondrial membrane in the matrix (Gamble and Lehninger, 1973; Clarke, 1976).

Previous studies have observed whole mitochondria (Swift and Hruban, 1964; Pfeifer, 1979; Marzella and Gloumann, 1987) or mitochondrial proteins (Vargas *et al.*, 1987; Knecht *et al.*, 1988; Knecht *et al.*, 1990) within lysosomes suggesting that mitochondria are degraded within autophagic vacuoles. Initial evidence suggested that there was a cooperation of lysosomes and inner mitochondrial membrane in the inactivation of CPS 1 and mitochondrial ATPase (Solar *et al.*, 1980). Recently, Vargas *et al.*, (1990) suggested that lysosomal proteinases can release a factor(s) (proteinase(s)) from the mitochondrial inner membrane thereby accelerating the proteolysis of CPS 1 and inactivating CPS 1. Interestingly, ATP and especially AGA accelerate the degradation of CPS 1 by the factor(s) liberated from the inner mitochondrial membrane (Vargas *et al.*, 1990). It was previously demonstrated that AGA activates CPS 1 inducing conformational changes (Rubio *et al.*, 1983) exposing

essential thiol (SH) groups (Novoa *et al.*, 1966; Marshall and Fahien, 1985) and its ATP-binding site (Rubio *et al.*, 1983) leaving it very sensitive to thermal (Caravara and Grisolia, 1959; Fahien *et al.*, 1964), oxidative (Alonso and Rubio, 1987) and proteolytic (Guadlajara *et al.*, 1987; Marshall and Fahien, 1988) inactivation, therefore supporting their observation.

Therefore, CPS 1 could become palmitoylated in the matrix of the mitochondria and when CPS 1 is palmitoylated it is inactivated. It then becomes transported or targeted to the inner mitochondrial membrane where it has been shown previously to interact. At the inner mitochondrial membrane a lysosomal proteinase(s) releases a probable proteinase(s) (large molecular weight) accelerating the degradation of CPS 1.

4.5 Future Experiments

In summary, further experimentation is required to understand the molecular basis of the fatty acylation of CPS 1 and GluDH. Identification of the site of the acylated cysteine residue(s) is of utmost importance. A widely employed technique used, is the substitution, using site directed mutagenesis, of the suspected acylated cysteine residue with a non-thiol amino acid such as alanine, serine or glycine, which cannot act as an acceptor of fatty acids by thioesterification (Rose *et al.*, 1984). Berthiaume *et al.*, (1994) reported that covalent fatty acylation of MMSDH occurs on its active site cysteine residue 319. CPS 1 has 21 free cysteine residues distributed throughout the protein (Clarke, 1976) but due to the similarity between a 17 amino acid sequence surrounding Cys1327 of CPS 1 and the fatty acylated active site cysteine 319 of

MMSDH, indicate that this cysteine may be a good candidate for fatty acylation (Table 4.1). Also, Cys1327 and Cys1337 have previously shown increased reactivity of thiol groups in the presence of the allosteric activator, AGA, and these sites are protected against any modification by Mg^{2+} and ATP (Geshwill and Lumper, 1989). In contrast to MMSDH, the catalytic mechanism and regulation of GluDH are rather complex (Hudson and Daniel, 1993) and may therefore complicate the identification of the fatty acylated cysteine(s). Fortunately, GluDH has only six cysteine residues (Amuro *et al.*, 1989). Interestingly, a radioactive azido-ATP analog was shown to be cross-linked to Cys319 of the nucleotide negative regulatory site of bovine liver GluDH (Ozturk and Coleman, 1991). Fatty acylation of this Cys319 in the negative regulatory site could thus represent an interesting variation on the theme of regulation by active site fatty acylation.

Along with Berthiaume *et al.*, (1994) we postulated that fatty acids could regulate the enzymatic activity of some key mitochondrial enzymes. Therefore, there must be a regulatory role inside the mitochondrial matrix for fatty acyl-CoAs with a chain length greater than 10 carbons, since short chain fatty acids (<10 carbons) can diffuse freely across the mitochondrial membranes (Schulz, 1985; Bremer, 1983). Hence, it is important to characterize the fatty acyl-CoA chain length specificity of CPS 1 and GluDH. Long chain fatty acyl CoAs (>8 carbons) inhibited fatty acylation of bovine liver MMSDH with maximal binding inhibition occurring with palmitoyl-CoA (Berthiaume *et al.*, 1994). No effect should be observed with fatty acyl-CoAs of less than eight carbons again, since

short chain fatty acids (<10 carbons) can diffuse freely across the mitochondrial membranes (Schulz, 1985; Bremer, 1983).

It is also important to determine the reversibility of the fatty acylation for CPS 1 and GluDH to support the reversibility proposed in our model. It has been reported that the rate of transfer of the fatty acid from the acyl-CoA to the acceptor protein or to specific cysteine-containing peptides is lower than that observed *in vivo* (Bizzozero and Good, 1991). In our studies, acylation of CPS 1 and GluDH seem to occur nonenzymatically, therefore we may observe lower rates of transfer of the fatty acid.

Table 4.1 Alignment of the sequence surrounding the active cysteine residue in bovine liver MMSDH with a potential fatty acylated cysteine residue in rat liver CPS 1.

```
MMSDH:   A G Q R C[319] - M A L S T - - A I L V G E
CPS I:   P I L R C[1327] E M A - S T G E V A C F G E
         o o o * *     * *   * *     o o o o * *
```

LEGEND:

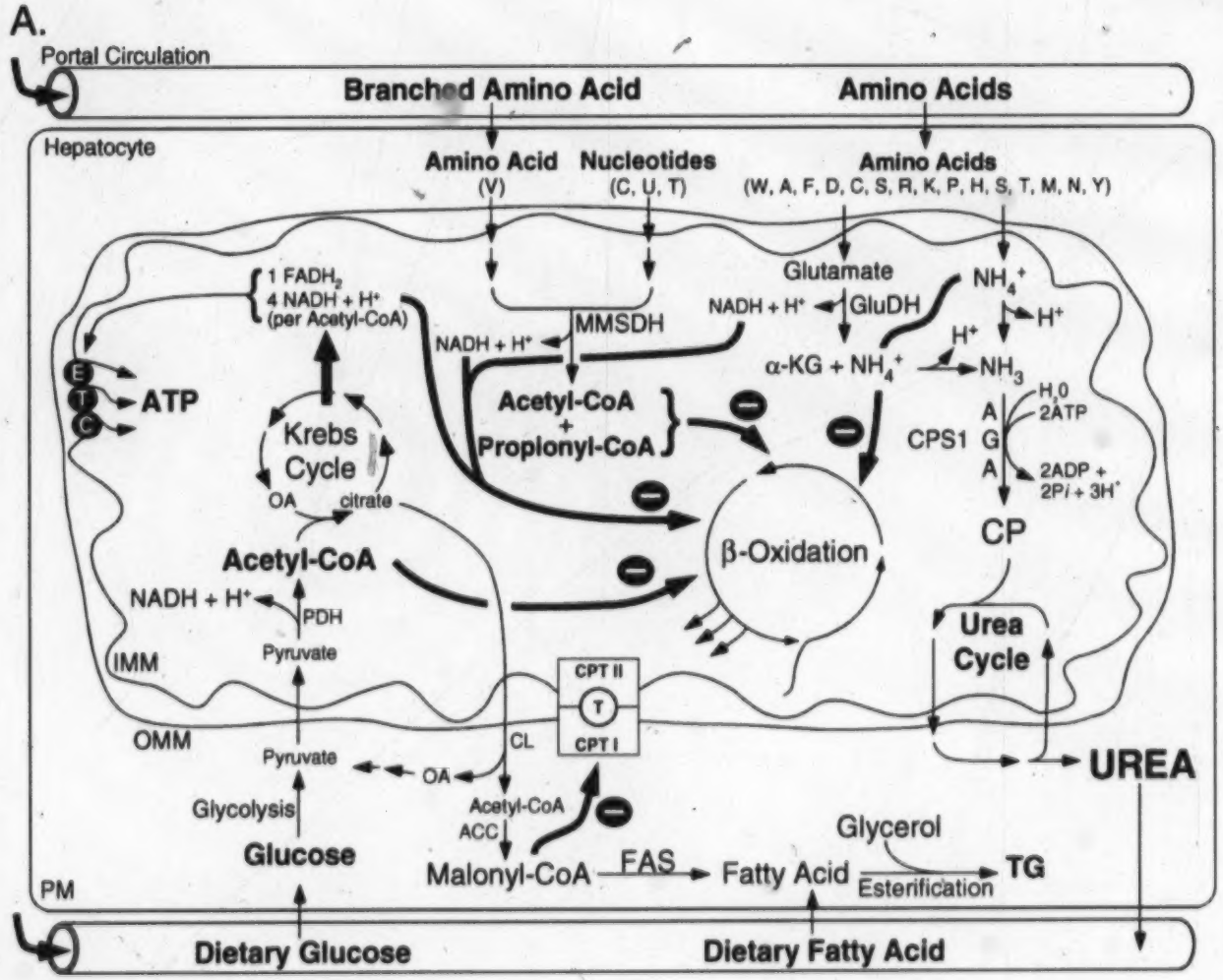
o: conserved amino acid substitution

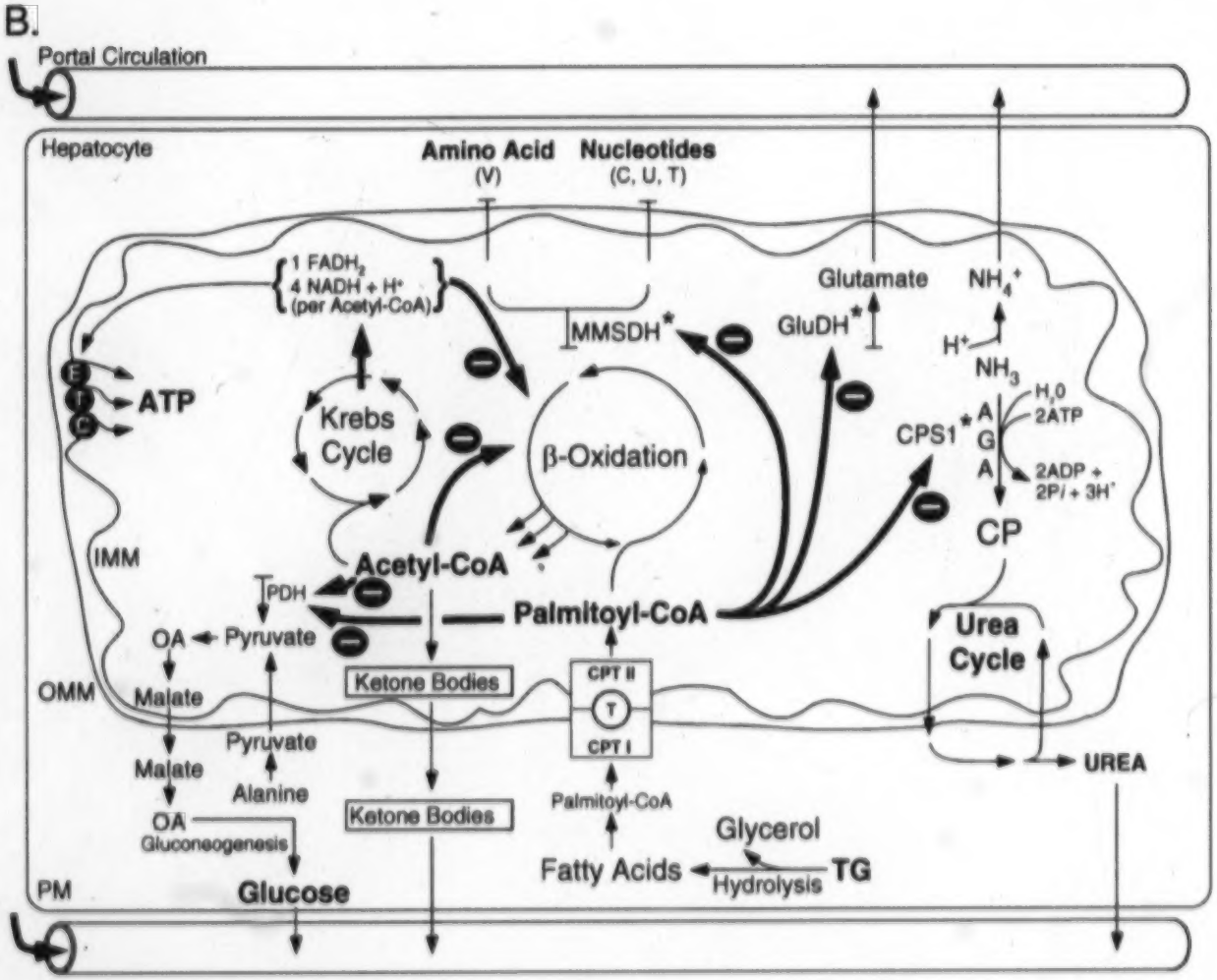
*: amino acid identity

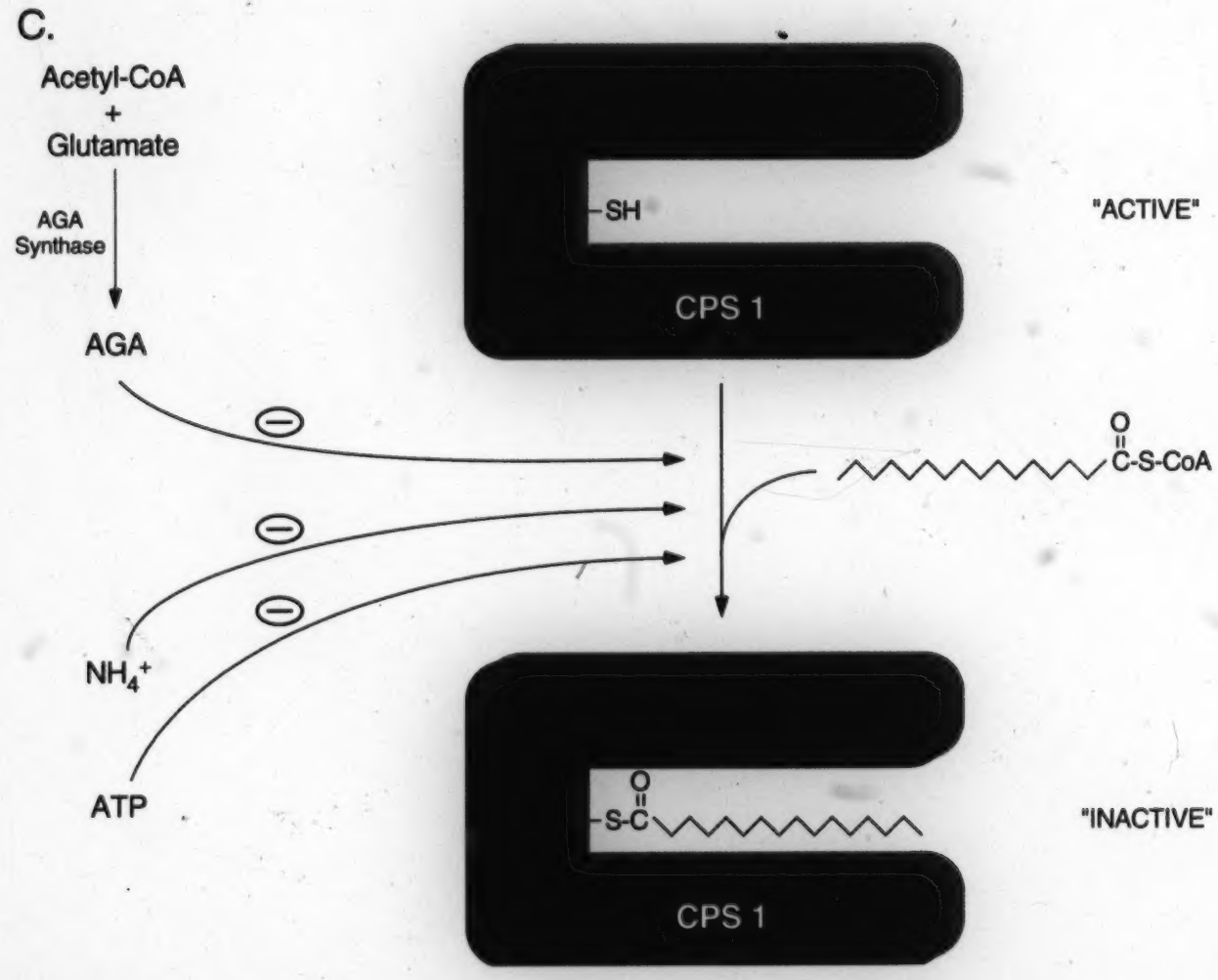
Fig. 4.1 Model depicting a putative role for mitochondrial protein fatty acylation in nitrogen sparing in the liver.

Metabolic cross-talk between predominant catabolic pathways in the mitochondria in the fed (**A**) and fasted (**B**) states. Bold arrows indicate documented inhibitions of catabolic cycles or enzymes by given metabolites. Mitochondrial fatty acylated enzymes are represented by an asterisk. The model summarizes observations from the references of Moore *et al.*, 1994; Kawaguchi and Bloch, 1976; Berthiaume *et al.*, 1994; Deichaite *et al.*, 1993; Moran *et al*,. 1994; Salway, 1994; Cahill, 1976a; Owen *et al.*, 1969; Owen *et al.*, 1967; Cahill, *et al.*, 1981; and Ishikawa *et al.*, 1976. In (**C**), a potential safety mechanism is described to prevent complete inhibition of CPS 1 and toxic accumulation ammonia.

Abbreviations are as follows: AGA, *N* -acetylglutamate; PDH, pyruvate dehydrogenase; ETC, electron transport chain; TG, triglyceride; OMM and IM, outer and inner mitochondrial membrane; PM, plasma membrane; MMSDH, methylmalonyl semialdehyde dehydrogenase; CPS 1, carbamoyl phosphate synthetase 1; GluDH, glutamate dehydrogenase; OA, oxaloacetate; CPT I and CPT II, carnitine palmitoyl transferase I and II; CL, citrate lyase; FAS, fatty acid synthetase; ACC, acetyl-CoA carboxylase.


A.
Portal Circulation
Branched Amino Acid
Amino Acids
Hepatocyte
Amino Acid (V)
Nucleotides (C, U, T)
Amino Acids (W, A, F, D, C, S, R, K, P, H, S, T, M, N, Y)
1 FADH2
4 NADH + H+
(per Acetyl-CoA)
MMSDH
NADH + H+
Glutamate
NADH + H+
GluDH
NH4+
H+
H+
α-KG + NH4+
NH3
ATP
Krebs Cycle
OA
citrate
Acetyl-CoA
+
Propionyl-CoA
H2O
2ATP
CPS1
A
G
A
2ADP + 2Pi + 3H+
CP
β-Oxidation
Acetyl-CoA
NADH + H+
PDH
Pyruvate
IMM
Urea Cycle
CPT II
T
CPT I
OMM
CL
UREA
Pyruvate
OA
Glycolysis
Acetyl-CoA
ACC
Glycerol
Glucose
Malonyl-CoA
FAS
Fatty Acid
Esterification
TG
PM
Dietary Glucose
Dietary Fatty Acid


B.
Portal Circulation
Hepatocyte
Amino Acid
(V)
Nucleotides
(C, U, T)
1 FADH2
4 NADH + H+
(per Acetyl-CoA)
MMSDH*
GluDH*
Glutamate
NH4+
H+
NH3
H2O
2ATP
2ADP +
2Pi + 3H+
CPS1*
CP
ATP
Krebs Cycle
β-Oxidation
IMM
Acetyl-CoA
PDH
Palmitoyl-CoA
OA
Pyruvate
Urea Cycle
OMM
Malate
Ketone Bodies
CPT II
T
CPT I
Pyruvate
Malate
Alanine
OA
Gluconeogenesis
Ketone Bodies
Palmitoyl-CoA
UREA
Glycerol
Fatty Acids
Hydrolysis
TG
PM
Glucose


C.
Acetyl-CoA
+
Glutamate
AGA
Synthase
AGA
SH
CPS 1
"ACTIVE"
O
C-S-CoA
NH_4^+
ATP
O
S-C
CPS 1
"INACTIVE"

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